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(54) Packaging systems for human recombinant adenovirus to be used in gene therapy

(57) The invention discloses novel means and methods for the generation of adenovirus vector. One method of the invention entails a method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of com-

bining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof. The invention further discloses nucleic acid molecules for generation adenovirus vectors.

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Description

[0001] The invention relates to the field of recombinant DNA technology, more in particular to the field of gene therapy. In particular the invention relates to gene therapy using materials derived from adenovirus, in particular human recombinant adenovirus. It especially relates to novel virus derived vectors and novel packaging cell lines for vectors based on adenoviruses.

[0002] Gene therapy is a recently developed concept for which a wide range of applications can be and have been envisaged. In gene therapy a molecule carrying genetic information is introduced into some or all cells of a host, as a result of which the genetic information is added to the host in a functional format. Included is the treatment of genetic disorders by providing the genetic information for supplementing a protein or other substance which is, through said genetic disorder, not present or at least present in insufficient amounts in the host, the treatment of tumors and (other) acquired disease such as (auto) immune diseases or infections, or other processes. The genetic information added may be a gene or a derivative of a gene, such as a cDNA, which encodes a protein. In this case the functional format means that the protein can be expressed by the machinery of the host cell. The genetic information can also be a sequence of nucleotides complementary to a sequence of nucleotides (be it DNA or RNA) present in the host cell. The functional format in this case is that the added DNA (nucleic acid) molecule or copies made thereof *in situ* are capable of base pairing with the complementary sequence present in the host cell.

[0003] Thus, there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards the removal or elimination of unwanted substances (organisms or cells) and the third directed towards application of a recombinant vaccine (tumors or foreign microorganisms).

[0004] For the purpose of gene therapy, adenoviruses carrying deletions have been proposed as suitable vehicles. Adenoviruses are non-enveloped DNA viruses. Gene transfer vectors derived from adenoviruses (so called adonoviral vectors) have a number of features that make them particularly useful for gene transfer for such purposes. For example, the biology of the adenoviruses is characterized in detail, the adenovirus is not associated with severe human pathology, the virus is extremely efficient in introducing its DNA into the host cell, the virus can infect a wide variety of cells and has a broad host range, the virus can be produced in large quantities with relative ease, and the virus can be rendered replication defective by deletions in the early region 1 (E1) of the viral genome.

[0005] The adenovirus (Ad) genome is a linear double-stranded DNA molecule of approximately 36000 base pairs with the 55-kDa terminal protein covalently bound to the 5' terminus of each strand. The Ad DNA contains identical Inverted Terminal Repeats (ITR) of about 100 base pairs with the exact length depending on the serotype. The viral origins of replication are located within the ITRs exactly at the genome ends. DNA synthesis occurs in two stages. First, the replication proceeds by strand displacement, generating a daughter duplex molecule and a parental displaced strand. The displaced strand is single stranded and can form a so called "panhandle" intermediate, which allows replication initiation and generation of a daughter duplex molecule. Alternatively, replication may proceed from both ends of the genome simultaneously, obviating the requirement to form the panhandle structure. The replication is summarized in Figure 14 adapted from Lechner *et al*, (1977) *J. Mol. Biol.* 174:493-510.

[0006] During the productive infection cycle, the viral genes are expressed in two phases: the early phase, which is the period up to viral DNA replication, and the late phase, which coincides with the initiation of viral DNA replication. During the early phase only the early gene products, encoded by regions E1, E2, E3 and E4, are expressed, which carry out a number of functions that prepare the cell for synthesis of viral structural proteins (Berk, A. J. (1986) *Ann. Rev. Genet.* 20:45-79). During the late phase the late viral gene products are expressed in addition to the early gene products and host cell DNA and protein synthesis are shut off. Consequently, the cell becomes dedicated to the production of viral DNA and of viral structural proteins (Tooze, J. (1981) *DNA Tumor Viruses (revised)*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

[0007] The E1 region of adenovirus is the first region of adenovirus expressed after infection of the target cell. This region consists of two transcriptional units, the E1A and E1B genes, both of which are required for oncogenic transformation of primary (embryonal) rodent cultures. The main functions of the E1A gene products are to induce quiescent cells to enter the cell cycle and resume cellular DNA synthesis, and to transcriptionally activate the E1B gene and the other early regions (E2, E3 and E4) of the viral genome. Transfection of primary cells with the E1A gene alone can induce unlimited proliferation (immortalization), but does not result in complete transformation. However, expression of E1A in most cases results in induction of programmed cell death (apoptosis), and only occasionally immortalization is obtained (Jochemsen *et al*, (19987) *EMBO J.* 6:3399-3405). Co-expression of the E1B gene is required to prevent induction of apoptosis and for complete morphological transformation to occur. In established immortal cell lines, high level expression of E1A can cause complete transformation in the absence of E1B (Roberts *et al*, (1985) *J. Virol.* 56: 404-413).

[0008] The E1B encoded proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55 kD and E4 33kD proteins, which form a complex that is essentially localized in the nucleus, function in inhibiting

the synthesis of host proteins and in facilitating the expression of viral genes. Their main influence is to establish selective transport of viral mRNAs from the nucleus to the cytoplasm, concomitantly with the onset of the late phase of infection. The E1B 21 kD protein is important for correct temporal control of the productive infection cycle, thereby preventing premature death of the host cell before the virus life cycle has been completed. Mutant viruses incapable of expressing the E1B 21 kD gene product exhibit a shortened infection cycle that is accompanied by excessive degradation of host cell chromosomal DNA (deg-phenotype) and in an enhanced cytopathic effect (cyt-phenotype) (Telling *et al.*, (1994) *J. Virol.* 68:541-7). The *deg* and *cyt* phenotypes are suppressed when in addition the E1A gene is mutated, indicating that these phenotypes are a function of E1A (White *et al.*, (1988) *J. Virol.* 62:3445-3454). Furthermore, the E1B 21 kDa protein slows down the rate by which E1A switches on the other viral genes. It is not yet known through which mechanisms E1B 21 kD quenches these E1A dependent functions.

[0009] Vectors derived from human adenoviruses, in which at least the E1 region has been deleted and replaced by a gene of interest, have been used extensively for gene therapy experiments in the pre-clinical and clinical phase, and all adenovirus vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Stratford-Perricaudet *et al.*, (1991) pp. 51-61. In O. Cohen-Adenaur, and M. Boiron (Eds). *Human Gene Transfer*, John Libbey Eurotext).

[0010] In contrast to, for example, retroviruses, adenoviruses do not integrate into the host cell genome, are able to infect non-dividing cells and are able to efficiently transfer recombinant genes *in vivo* (Brody *et al.*, (1994) *Ann NY Acad. Sci.* 716:90-101). These features make adenoviruses attractive candidates for *in vivo* gene transfer of, for example, suicide or cytokine genes into tumor cells. However, a problem associated with current recombinant adenovirus technology is the possibility of unwanted generation of replication competent adenovirus (RCA) during the production of recombinant adenovirus (Lochmüller *et al.*, (1994) *Hum. Gene Ther.* 5:1485-1492; Imler *et al.*, (1996) *Gene Ther.* 3:75-84). This is caused by homologous recombination between overlapping sequences from the recombinant vector and the adenovirus constructs present in the complementing cell line, such as the 293 cells (Graham *et al.*, (1977) *J. Gen. Virol.* 36:59-72). RCA in batches to be used in clinical trials is unwanted because RCA i) will replicate in an uncontrolled fashion; ii) can complement replication defective recombinant adenovirus, causing uncontrolled multiplication of the recombinant adenovirus; and iii) batches containing RCA induce significant tissue damage and hence strong pathological side effects (Lochmüller *et al.*, (1994) *Hum. Gene Ther.* 5:1485-1492). Therefore, batches to be used in clinical trials should be proven, free of RCA (Ostrove, J. M. (1994) *Cancer Gene Ther.* 1:125-131).

[0011] One of the additional problems associated with the use of recombinant adenovirus vectors is the host defense reaction against treatment with adenovirus. Briefly, recombinant adenoviruses are deleted for the E1 region (see above). The adenovirus E1 products trigger the transcription of the other early genes (E2, E3, E4), which consequently activate expression of the late virus genes. Therefore, it was generally thought that E1 deleted vectors would not express any other adenovirus genes. However, recently it has been demonstrated that some cell types are able to express adenovirus genes in the absence of E1 sequences. This indicates, that some cell types possess the machinery to drive transcription of adenovirus genes. In particular, it was demonstrated that such cells synthesize E2A and late adenovirus proteins. In a gene therapy setting, this means that transfer of the therapeutic recombinant gene to somatic cells not only results in expression of the therapeutic protein but may also result in the synthesis of viral proteins. Cells that express adenoviral proteins are recognized and killed by cytotoxic T Lymphocytes, which both eradicates the transduced cells and causes inflammation (Bout *et al.*, (1994a) *Gene Therapy* 1:385-394; Engelhardt *et al.*, (1993) *Human Gene Therapy* 4:759-769; Simon *et al.*, (1993) *Human Gene Therapy* 4:771-780). As this adverse reaction hampers gene therapy, several solutions to this problem have been suggested, such as using immunosuppressive agents after treatment, retaining the adenovirus E3 region in the recombinant vector (see patent application EP 952022 1B) or using ts mutants of human adenovirus, which have a point mutation in the E2A region (patent WO/28938). However, these strategies to circumvent the immune response have their limitations. The use of ts mutant recombinant adenovirus diminishes the immune response to some extent, but is less effective in preventing pathological responses in the lungs (Engelhardt *et al.*, (1994a) *Human Gene Ther.* 5:1217-1229). The E2A protein may induce an immune response by itself and it plays a pivotal role in the switch to the synthesis of late adenovirus proteins. Therefore, it is attractive to make recombinant adenoviruses which are mutated in the E2 region, rendering it temperature sensitive (ts), as has been claimed in patent application WO/28938. A major drawback of this system is the fact that, although the E2 protein is unstable at the non-permissive temperature, the immunogenic protein is still synthesized. In addition, it is to be expected that the unstable protein does activate late gene expression, albeit to a low extent. ts125 mutant recombinant adenoviruses have been tested, and prolonged recombinant gene expression was reported (Yang *et al.*, (1994b) *Nat. Genet.* 7:362-369; Engelhardt *et al.*, (1994a) *Hum. Gene Ther.* 5:1217-1229; Engelhardt *et al.*, (1994b) *Proc. Natl. Acad. Sci. USA* 91:6196-200; Yang *et al.*, (1995) *J. Virol.* 69:2004-2015). However, pathology in the lungs of cotton rats was still high (Engelhardt *et al.*, (1994a) *Human Gene Ther.* 5:1217-1229), indicating that the use of ts mutants results in only a partial improvement in recombinant adenovirus technology. Others (Fang *et al.*, (1996) *Gene Ther.* 3:217-222) did not observe prolonged gene expression in mice and dogs using ts125 recombinant adenovirus. An additional difficulty associated with the use of ts125 mutant adenoviruses is that a high frequency of reversion is

observed. These revertants are either real revertants or the result of second site mutations (Kruijer *et al.*, (1983) *Virology* 124:425-433; Nicolas *et al.*, (1981) *Virology* 108:521-524). Both types of revertants have an E2A protein that functions at normal temperature and therefore have similar toxicity as the wild-type virus.

[0012] E1 deleted recombinant adenoviruses are usually made by one of the following method. In the first method, adenovirus DNA, be it wild type (wt) or E1 and/or E3 deleted, is digested with a restriction enzyme e.g. *Clal*, to remove the left ITR, packaging signal and at least part of the E1 sequences and the remaining adenovirus genome fragment (1) is purified. Cotransfection of (1) with a linearized adapter construct (2) containing the left ITR, packaging signal, an expression cassette with the gene of interest and adenovirus sequences overlapping with (1) in a cell line complementing for E1 functions (packaging cell line) will give rise to recombinant adenovirus particles by intra-cellular homologous recombination. Alternatively, an adapter construct (3) containing the left ITR, packaging signal, and an expression cassette with the gene of interest is such that it can be ligated to the adenovirus DNA fragment (1) followed by transfection into packaging cells. The disadvantage of these methods is that the purification of (1) is laborious and that incomplete digestion of wt DNA results in introduction of wt adenovirus into the culture leading to contamination. An approach to circumvent this problem has been by the construction of clone pHBG10 described by Bett *et al.*, (1994) *Natl. Acad. Sci. USA* 91:8802-8806. This plasmid clone contains Ad5 sequences with a deletion of the packaging signal and part of the E1 region and with the viral ITRs attached to each other. However, this clone comprises adenovirus sequences that are also present in E1-complementing cell lines, including those of the present invention (see EP 95201611.1). Furthermore, since the ITRs are attached to each other the clone cannot be linearized, resulting in less efficient recombination with the E1 substitution plasmid.

[0013] In the second method, the recombinant adenoviruses is constructed either by homologous recombination in bacteria (Chartier *et al.*, (1996) *J. Virol.* 70, No. 7:4805-4810; Crouzet *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* 94:1414-1419) or by cloning into cosmid vectors (Fu *et al.*, (1997) *Hum. Gene Ther.* 8:1321-1330) and subsequent transfection into an E1 complementing cell line. The disadvantage of this method is that it demands extensive analysis of each generated clone (~35 kb) by restriction enzyme digestion before transfection to exclude deletions that occurred due to recombination in the bacteria. In addition, the use of cloned adenovirus sequences does not solve the problem of sequence overlap between commonly used packaging cells and recombinant viruses leading to production of RCA during propagation.

[0014] A third method that is used is a two-step gene replacement technique in yeast, starting with a complete adenovirus genome (Ad2; Ketner *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:6186-6190) cloned in a Yeast Artificial Chromosome (YAC) and a plasmid containing adeno sequences to target a specific region in the YAC clone, an expression cassette for the gene of interest and a positive and negative selectable marker. This method requires yeast technology and extensive analysis of each new recombinant clone (even more cumbersome than the above described method, due to the large size of YACS).

[0015] A fourth method uses a cosmid clone (pAdox1w; Miyake *et al.*, (1996) *Medical Sciences* 93:1320-1324) that carries the Ad5 sequence with deletions in the E1 and E3 sequences. This clone has a unique restriction site replacing part of the E1 region that allows insertion of a foreign expression cassette. For the generation of recombinant adenoviruses, a DNA-terminal protein complex (DNA-TPC) is isolated from cells infected with a replication competent adenovirus Ad-dIX (wt Ad5 with an *XbaI* deletion in the E3 region). This DNA is digested with *EcoT221* to remove the 5' part of the DNA and cotransfected with the cosmid cloned into E1 complementing cells. Intracellular recombination generates the recombinant virus (Miyake *et al.*, (1996) *Medical Sciences* 93:1320-1324). This method has the disadvantage that replication competent viral DNA is used and that the E1 deletion in the cosmid clone is not enough to remove all overlap with E1 sequences in currently used packaging cell lines including those used in the present invention. Thus, current methods to generate RCA-free recombinant adenoviruses have several disadvantages, including the risk of introducing wild-type viruses in the culture, instability of cloned adenovirus sequences, the necessity to check the complete ~35 kb recombinant clone by restriction analysis for each new virus to be generated, and the system being suitable only for E1 deleted recombinant adenoviruses and much more laborious for use with recombinant adenoviruses comprising E3 substitutions. Furthermore, despite the use of cloned adenovirus DNA in some of the methods, extensive overlap with adenovirus sequences present in commonly used packaging cells like 293 and 911 cells does not solve the problem of appearance of RCA due to homologous recombination during propagation of the virus. Therefore, a need persists for methods and means to produce RCA-free recombinant adenovirus preparations that solve the disadvantages of prior art methods and means discussed above. Gene addition is currently by far the most widely applied gene therapy technique. This is mainly due to the fact that a) homologous recombination is very inefficient and b) for homologous recombination relatively large DNA fragments are required for which no suitable vector systems were available. Thus there is currently an unmet need for vector systems that efficiently introduce large nucleic acid molecules into mammalian cells.

[0016] Recombinant adenoviruses are able to efficiently transfer recombinant genes to the rat liver and airway epithelium of rhesus monkeys (Bout *et al.*, (1994b) *Human Gene Therapy* 5:3-10; Bout *et al.*, (1994a) *Gene Therapy* 1:385-394). In addition, (Vincent *et al.*, (1996) *J. Neurosurg* 85:648-654; Vincent *et al.*, (1996b) *Hum. Gene Ther.* 7:

197-205) and others (see for example Haddada *et al.* (1993) *Hum. Gene Ther.* 4:703-11) have observed an efficient *in vivo* adenovirus mediated gene transfer to a variety of tumor cells *in vitro* and to solid tumors in animal models (lung tumors, glioma) and human xenografts in immunodeficient mice (lung) *in vivo* (reviewed by Blaese *et al.*, *Cancer Gene Ther.* 2:291-297)

5 [0017] Generation of minimal adenovirus vectors has been disclosed in WO 94/12649. The method described exploits the function of the protein IX for the packaging of minimal adenovirus vectors (Pseudo Adenoviral Vectors (PAV) in the terminology of WO 94/12649). PAVs are produced by cloning an expression plasmid with the gene of interest between the left-hand (including the sequences required for encapsulation) and the right-hand adenoviral ITRs. The PAV is propagated in the presence of a helper virus. Encapsidation of the PAV is preferred compared to the helper virus because the helper virus is partially defective for packaging (either by virtue of mutations in the packaging signal or by virtue of its size (virus genomes greater than 37.5 kb package inefficiently)). In addition, the authors propose that in the absence of the protein IX gene the PAV will be preferentially packaged. However, neither of these mechanisms appear to be sufficiently restrictive to allow packaging of only PAVs/minimal vectors. The mutations proposed in the packaging signal diminish packaging, but do not provide an absolute block as the same packaging activity is required to propagate the helper virus. Also neither an increase in the size of the helper virus nor the mutation of the protein IX gene will ensure that PAV is packaged exclusively. Thus, the method described in WO 94/12649 is unlikely to be useful for the production of helper-free stocks of minimal adenovirus vectors/PAVs.

10 [0018] Novel compositions and methods are provided for producing recombinant adenoviruses, not only E1-deleted but also minimal adenoviruses which are free of replication competent adenoviruses. The compositions include constructs suitable for the generation of double insert viruses. The system provided by the invention to generate for example E1 deleted adenoviruses consists of two nucleic acid molecules of which a first is a relatively small and easy to manipulate adapter plasmid containing at least, in an operable configuration, the left ITR, the packaging signal, if desired an expression cassette with the nucleic acid molecule of interest and sequences homologous to a part of a second molecule that comprises at least one partially overlapping nucleic acid molecule that comprise at least the right ITR and preferably further comprises adenovirus sequences encoding adenovirus capsid proteins, and the packaging cells of the invention described *infra*. Co-transfection of said nucleic acid molecules into the packaging cells allows the welding together of said nucleic acid molecules preferably through essentially one homologous recombination between the overlapping sequences in said nucleic acid molecules. Homologous recombination generates a recombinant viral DNA that is able to replicate and propagate on the packaging cells. The nucleic acid molecules preferably have no sequence overlap with complementing sequences in packaging cells that can lead to the formation of replication competent adenovirus (RCA). Preferably, at least one of the ITRs on the nucleic acid molecules is flanked by a restriction enzyme recognition site not present in the adenoviral sequences so that the ITR can be made essentially free from vector sequences by digestion of the DNA with that restriction enzyme. In this way initiation of replication occurs more efficiently. The system provided by the present invention also greatly facilitates the production of RCA-free recombinant adenoviruses with further modifications in the adenovirus genome, including but not limited to modifications in the coding regions for the E4 region proteins, hexon, penton base protein or fiber protein or E2A protein.

35 [0019] The following embodiments should be understood to be read in the light of the combination of adapter plasmid and the large nucleic acid to be welded together therewith.

40 [0020] In one aspect of the invention the problem with RCA production is solved in that we have developed packaging cells that have no overlapping sequences with a new basic vector and thus are suited for safe large scale production of recombinant adenoviruses.

[0021] In another aspect of the present invention we therefore delete E2A coding sequences from the recombinant adenovirus genome and transfect these E2A sequences into the (packaging) cell lines containing E1 sequences to complement recombinant adenovirus vectors.

45 [0022] Major hurdles in this approach are a) that E2A should be expressed to very high levels and b) that E2A protein is very toxic to cells.

50 [0023] The current invention in yet another aspect therefore discloses use of the ts125 mutant E2A gene, which produces a protein that is not able to bind DNA sequences at the non permissive temperature. High levels of this protein may be maintained in the cells (because it is not toxic at this temperature) until the switch to the permissive temperature is made. This can be combined with placing the mutant E2A gene under the direction of an inducible promoter, such as for instance tet, methallothionein, steroid inducible promoter, retinoic acid β -receptor or other inducible systems. However, in yet another aspect of the invention, the use of an inducible promoter to control the moment of production of toxic wild-type E2A is disclosed.

55 [0024] Two salient additional advantages of E2A-deleted recombinant adenovirus are the increased capacity to harbor heterologous sequences and the permanent selection for cells that express the mutant E2A. This second advantage relates to the high frequency of reversion of ts125 mutation: when reversion occurs in a cell line harboring ts125 E2A, this will be lethal to the cell. Therefore, there is a permanent selection for those cells that express the ts125 mutant E2A protein. In addition, as we in one aspect of the invention generate E2A-deleted recombinant adenovirus, we will

not have the problem of reversion in our adenoviruses.

[0025] In yet another aspect of the invention as a further improvement the use of non-human cell lines as packaging cell lines is disclosed.

[0026] For GMP production of clinical batches of recombinant viruses it is desirable to use a cell line that has been used widely for production of other biotechnology products. Most of the latter cell lines are from monkey origin, which have been used to produce, for example vaccines.

[0027] These cells cannot be used directly for the production of recombinant human adenovirus, as human adenovirus cannot replicate in cells of monkey origin or only replicates at low levels. A block in the switch of early to late phase of adenovirus lytic cycle underlies the defective replication. However, host range (hr) mutations in the human adenovirus genome are described (hr400 - 404) which allow replication of human viruses in monkey cells. These mutations reside in the gene encoding E2A protein (Klessig *et al.*, (1979) *Cell* 17:957-966; Klessig *et al.*, (1984) *Virus Res.* 1:169-188; Rice *et al.*, (1985) *J. Virol.* 56:767-773) (Klessig *et al.*, (1984) *Virus Res.* 1:169-188). Moreover, mutant viruses have been described that harbor both the hr and temperature-sensitive ts125 phenotype (Brough *et al.*, (1965) *J. Virol.* 55, 206-212; Rice *et al.*, (1985) *J. Virol.* 56:767-778).

[0028] We therefore generate packaging cell lines of monkey origin (e.g., VERO, CV1) that harbor:

- a) E1 sequences, to allow replication of E1/E2 defective adenoviruses, and
- b) E2A sequences, containing the hr mutation and the ts125 mutation named ts400 (Brough *et al.*, (1985) *J. Virol.* 55:206-212; Rice *et al.*, (1985) *J. Virol.* 56:767-778 to prevent cell death by E2A overexpression, and/or
- c) E2A sequences, just containing the hr mutation, under the control of an inducible promoter, and/or
- d) E2A sequences, containing the hr mutation and the ts125 mutation (ts400), under the control of an inducible promoter.

[0029] Furthermore, we disclose the construction of novel and improved combinations of (novel and improved) packaging cell lines and (novel and improved) recombinant adenovirus vectors.

[0030] We provide:

- 1) A novel packaging cell line derived from diploid human embryonic retinoblasts (HER) that harbors nt.80-5788 of the Ad5 genome. This cell line, named 911, deposited under No. 95062101 at the ECACC, has many characteristics that make it superior to the commonly used 293 cells (Fallaux *et al.*, (1996) *Hum. Gene Ther.* 7:215-222).
- 2) Novel packaging cell lines that express just E1A genes and not E1B genes. Established cell lines (and not human diploid cells of which 293 and 911 cells are derived) are able to express E1A to high levels without undergoing apoptotic cell death, as occurs in human diploid cells that express E1A in the absence of E1B. Such cell lines are able to transcomplement E1B-defective recombinant adenoviruses, because viruses mutated for E1B 21 kD protein are able to complete viral replication even faster than wild-type adenoviruses (Telling *et al.*, (1994) *J. Virol.* 68:541-7). The constructs are described in detail below, and graphically represented in Figures 1-5. The constructs are transfected into the different established cell lines and are selected for high expression of E1A. This is done by operatively linking a selectable marker gene (e.g. NEO gene) directly to the E1B promoter. The E1B promoter is transcriptionally activated by the E1A gene product and therefore resistance to the selective agent (e.g. G418 in the case NEO is used as the selection marker) results in direct selection for desired expression of the E1A gene.
- 3) Packaging constructs that are mutated or deleted for E1B 21 kD, but just express the 55 kD protein.
- 4) Packaging constructs to be used for generation of complementing packaging cell lines from diploid cells (not exclusively of human origin) without the need of selection with marker genes. These cells are immortalized by expression of E1A. However, in this particular case expression of E1B is essential to prevent apoptosis induced by E1A proteins. Selection of E1 expressing cells is achieved by selection for focus formation (immortalization), as described for 293 cells (Graham *et al.*, (1977) *J. Gen. Virol.* 36:59-72) and 911 cells (Fallaux *et al.*, (1996) *Hum. Gene Ther.* 7:215-222), that are E1 transformed human embryonic kidney (HEK) cells and human embryonic retinoblasts (HER), respectively.
- 5) After transfection of HER cells with construct pIG.E1A.E1B (Fig. 4), seven independent cell lines could be established. These cell lines were designated PER.C1, PER.C3, PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9. PER denotes PGK-E1-Retinoblasts. These cell lines express E1A and E1B proteins, are stable (e.g. PER.C6 for more than 57 passages) and complement E1-defective adenovirus vectors. Yields of recombinant adenovirus obtained on PER cells are a little higher than obtained on 293 cells. One of these cell lines (PER.C6) has been deposited at the ECACC under number 96022940.
- 6) New adenovirus vectors with extended E1 deletions (deletion nt.459-3510). Those viral vectors lack sequences homologous to E1 sequences in said packaging cell lines. These adenoviral vectors contain pIX promoter sequences and the pIX gene, as pIX (from its natural promoter sequences) can only be expressed from the vector

and not by packaging cells (Matsui *et al.*, (1986) *Mol. Cell Biol.* 6 4149-4154, Hoeben and Fallaux, pers. comm., Imler *et al.*, (1996) *Gene Ther.* 3:75-84)

7) E2A expressing packaging cell lines preferably based on either E1A expressing established cell lines or E1A + E1B expressing diploid cells (see under 2-4). E2A expression is either under the control of an inducible promoter or the E2A ts125 mutant is driven by either an inducible or a constitutive promoter.

8) Recombinant adenovirus vectors as described before (see 6) but carrying an additional deletion of E2A sequences.

9) Adenovirus packaging cells from monkey origin that are able to transcomplement E1-defective recombinant adenoviruses. They are preferably co-transfected with pIG.E1A.E1B and pIG.NEO, and selected for NEO resistance. Such cells expressing E1A and E1B are able to transcomplement E1 defective recombinant human adenoviruses, but will do so inefficiently because of a block of the synthesis of late adenovirus proteins in cells of monkey origin (Klessig *et al.*, (1979) *Cell* 17:957-966). To overcome this problem, we generate recombinant adenoviruses that harbor a host-range mutation in the E2A gene, allowing human adenoviruses to replicate in monkey cells. Such viruses are generated as described in Figure 12., except DNA from a hr-mutant is used for homologous recombination.

10) Adenovirus packaging cells from monkey origin as described under 9, except that they will also be co-transfected with E2A sequences harboring the hr mutation. This allows replication of human adenoviruses lacking E1 and E2A (see under 8). E2A in these cell lines is either under the control of an inducible promoter or the tsE2A mutant is used. In the latter case, the E2A gene will thus carry both the ts mutation and the hr mutation (derived from ts400). Replication competent human adenoviruses have been described that harbor both mutations (Brough *et al.*, *J. Virol.* 55:206-212; Rice *et al.*, (1985) *J. Virol.* 56:767-778).

[0031] Furthermore, the present invention in one aspect provides new cosmid and plasmid vectors containing large fragments of the adenovirus genome and an improved method for the generation of recombinant adenoviral vectors by making use of these cloned adenovirus sequences

[0032] Accordingly, the present invention provides a new system to generate recombinant adenoviruses that is fast, highly flexible, reliable and only requires standard cloning technology. The new system is surprisingly efficient in generating recombinant adenoviruses. In combination with the packaging cells of the invention it ensures RCA-free generation and propagation of recombinant adenoviruses. The above listed problems associated with current methods to generate recombinant adenoviruses are in one aspect solved by using a functional combination of cloned adenovirus sequences and an intra-cellular homologous recombination in suitable packaging cells.

[0033] Accordingly, the present invention provides methods and means to efficiently generate and produce vectors that are able to harbor very large fragments of (genomic) DNA. Vectors of the invention can be safely produced to very high titers and are able to transduce mammalian cells, including human cells, with high efficiency, thereby favoring homologous recombination with (genomic) DNA molecules present in said mammalian cells, due to the high numbers of introduced DNA molecules and their large homologous overlap with the target DNA molecules for recombination. In one aspect vectors according to the invention are based on adenoviral vectors derived from an adenoviral genome, from which as much as possible of the adenoviral genome is deleted except for the ITR sequences and the sequences needed *in cis* for packaging (minimal adenovirus vectors). Such vectors can accommodate up to 38 kb of foreign (genomic) DNA.

[0034] Minimal adenoviral vectors with large genomic sequences functioning as gene replacement vectors can be generated efficiently using the plasmid-based intracellular PCR system disclosed *infra*, thereby avoiding the need of contaminating helper viruses. In addition, we disclose an alternative way of producing minimal adenoviral vectors without the need for helper viruses. Replication and packaging of the minimal adenoviral vectors with large inserts can also be achieved by using them in combination with a complementing molecule containing all parts of the adenovirus genome that are required for replication and packaging except for the packaging signal and E1 sequences. Such a complementing molecule not necessarily replicates by the virtue of the adenoviral replication machinery. It may, for example, be cloned on a plasmid that also contains the SV40 origin of replication. Transfection of this DNA together with the minimal adenoviral vector in a E1-containing packaging cell that also (inducibly) expresses the SV40 Large T protein will lead to replication of the adenovirus molecule and expression of adenoviral proteins. The latter will then initiate replication and packaging of the minimal adenoviral vectors.

[0035] A further aspect of the invention provides otherwise improved adenovirus vectors, as well as novel strategies for generation and application of such vectors and a method for the intracellular amplification of linear DNA fragments in mammalian cells.

[0036] The so-called "minimal" adenovirus vectors according to the present invention retain at least a portion of the viral genome that is required for encapsulation of the genome into virus particles (the encapsulation signal), as well as at least one copy of at least a functional part or a derivative of the Inverted Terminal Repeat (ITR), that is DNA sequences derived from the termini of the linear adenovirus genome. The vectors according to the present invention

typically also contain a transgene linked to a promoter sequence to govern expression of the transgene. Packaging of the so-called minimal adenovirus vector can be achieved by co-infection with a helper virus or, alternatively, with a packaging deficient replicating helper system as described below.

[0037] Adenovirus-derived DNA fragments that can replicate in suitable cell lines and that may serve as a packaging deficient replicating helper system are generated as follows. These DNA fragments retain at least a portion of the transcribed region of the "late" transcription unit of the adenovirus genome and carry deletions in at least a portion of the E1 region and deletions in at least a portion of the encapsulation signal. In addition, these DNA fragments contain at least one copy of an inverted terminal repeat (ITR). At one terminus of the transfected DNA molecule an ITR is located. The other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3'terminal nucleotide of the hairpin structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion into a double-stranded form of at least a portion of the DNA molecule. Further replication initiating at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's and is larger than the original transfected DNA molecule (see Fig. 13). This molecule can replicate itself in the transfected cell by virtue of the adenovirus proteins encoded by the DNA molecule and the adenoviral and cellular proteins encoded by genes in the host cell genome. This DNA molecule cannot be encapsulated due to its large size (greater than 39000 base pairs) and/or due to the absence of a functional encapsulation signal. This DNA molecule is intended to serve as a helper for the production of defective adenovirus vectors in suitable cell lines.

[0038] The invention also comprises a method for the amplification of linear DNA fragments of variable size in suitable mammalian cells. These DNA fragments contain at least one copy of the ITR at one of the termini of the fragment. As described above, the other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3'terminal nucleotide of the hairpin-structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion of the displaced strand into a double stranded form of at least a portion of the DNA molecule. Further replication initiation at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's, which is larger than the original transfected DNA molecule. A DNA molecule that contains ITR sequences at both ends can replicate itself in transfected cells by virtue of the presence of at least the adenovirus E2 proteins (namely the DNA binding protein (DBP), the adenovirus DNA polymerase (Ad-pol), and the preterminal protein (pTP)). The required proteins may be expressed from adenovirus genes on the DNA molecule itself, from adenovirus E2 genes integrated in the host-cell genome, or from a replicating helper fragment as described above.

[0039] Several groups have shown that the presence of ITR sequences at the end of DNA molecules are sufficient to generate adenovirus minichromosomes that can replicate, if the adenovirus-proteins required for replication are provided in trans e.g. by infection with a helper virus (Hu *et al.*, (1992) *Gene* 110:145-150); (Wang *et al.*, (1985) *in vivo. Nucl. Acids Res.* 13:5173-5187); Hay *et al.*, (1984) *J. Mol. Biol.* 174:493-510). Hu *et al.*, (1992) *Gene* 110:145-150, observed the presence and replication of symmetrical adenovirus minichromosome-dimers after transfection of plasmids containing a single ITR. The authors were able to demonstrate that these dimeric minichromosomes arise after tail-to-tail ligation of the single ITR DNA molecules. In DNA extracted from defective adenovirus type 2 particles, dimeric molecules of various sizes have also been observed using electron-microscopy (Daniell (1976) *J. Virol.* 19:665-708). It was suggested that the incomplete genomes were formed by illegitimate recombination between different molecules and that variations in the position of the sequence at which the illegitimate base pairing occurred were responsible for the heterogeneous nature of the incomplete genomes. Based on this mechanism it was speculated that, in theory, defective molecules with a total length of up to two times the normal genome could be generated. Such molecules could contain duplicated sequences from either end of the genome. However, no DNA molecules larger than the full-length virus were found packaged in the defective particles (Daniell (1976) *J. Virol.* 19:665-708. This can be explained by the size-limitations that apply to the packaging. In addition, it was observed that in the virus particles DNA-molecules with a duplicated left-end predominated over those containing the right-end terminus (Daniell (1976) *J. Virol.* 19: 665-708). This is fully explained by the presence of the encapsulation signal near that left-end of the genome (Gräble *et al.*, (1990) *J. Virol.* 64:2047-2056; Gräble *et al.*, (1992) *J. Virol.* 66:723-731; Hearing *et al.*, (1967) *J. Virol.* 61: 2555-2558).

[0040] The major problems associated with the current adenovirus-derived vectors are:

- a) The strong immunogenicity of the virus particle.
- b) The expression of adenovirus genes that reside in the adenoviral vectors, resulting in a Cytotoxic T-cell response against the transduced cells.
- c) The low amount of heterologous sequences that can be accommodated in the current vectors (up to maximally approx. 8000 bp. of heterologous DNA).

d) The poor frequency and poor reliability of the methods and means for the generation of new adenovirus vectors.

[0041] Ad A) The strong immunogenicity of the adenovirus particle results in an immunological response of the host, even after a single administration of the adenoviral vector. As a result of the development of neutralizing antibodies, a subsequent administration of the virus will be less effective or even completely ineffective. However, a prolonged or persistent expression of the transferred genes will reduce the number of administrations required and may bypass the problem.

[0042] Ad B) Experiments performed by Wilson and collaborators have demonstrated that after adenovirus-mediated gene transfer into immunocompetent animals, the expression of the transgene gradually decreases and disappears approximately 2-4 weeks post-infection (Yang *et al.*, (1994a) *Proc. Natl. Acad. Sci USA* 91:4407-11; Yang *et al.*, (1994b) *Nat. Genet.* 7:362-369). This is caused by the development of a cytotoxic T-cell (CTL) response against the transduced cells. The CTLs were directed against adenovirus proteins expressed by the viral vectors. In the transduced cells synthesis of the adenovirus DNA-binding protein (the E2A-gene product), penton and fiber proteins (late-gene products) could be established. These adenovirus proteins, encoded by the viral vector, were expressed despite deletion of the E1 region. This demonstrates that deletion of the E1 region is not sufficient to completely prevent expression of the viral genes (Engelhardt *et al.*, (1994a) *Human Gene Ther.* 5:1217-1229).

[0043] Ad C) Studies by Graham and collaborators have demonstrated that adenoviruses are capable of encapsulating DNA of up to 105% of the normal genome size (Ben *et al.*, (1993) *J. Virol.* 67:5911-5921). Larger genomes tend to be unstable resulting in loss of DNA sequences during propagation of the virus. Combining deletions in the E1 and E3 regions of the viral genomes increases the maximum size of the foreign that can be encapsulated to approx. 8.3 kb. In addition, some sequences of the E4 region appear to be dispensable for virus growth (adding another 1.8 kb to the maximum encapsulation capacity). Also the E2A region can be deleted from the vector, when the E2A gene product is provided in trans in the encapsulation cell line, adding another 1.6 kb. It is, however, unlikely that the maximum capacity of foreign DNA can be significantly increased further than 12 kb.

[0044] We developed a new strategy for the generation and production of helperfree-stocks of recombinant adenovirus vectors that can accommodate up to 38 kb of foreign DNA. Only two functional ITR sequences, and sequences that can function as an encapsulation signal need to be part of the vector genome. Such vectors are called minimal adenovectors. The helper functions for the minimal adenovectors are provided in trans by encapsulation defective-replication competent DNA molecules that contain all the viral genes encoding the required gene products, with the exception of those genes that are present in the host-cell genome, or genes that reside in the vector genome.

Ad D) The generation of a new adenovirus vector with the means and methods in the prior art is possible. However, the prior art means and methods are not very efficient in generating adenovirus vector and moreover in the process of generating said adenovirus vector many other vectors or even replication competent adenovirus is produced necessitating a thorough and elaborate evaluation of the generated viruses. This is not a desired feature, specifically for clinical settings the presence of replication competent adenovirus is extremely undesired. In addition, specifically in settings where many different adenovirus vectors need to be generated, such as to produce expression libraries in adenovirus vectors for instance for use in high throughput screenings, the efficiency and the reliability of prior art adenovirus vector production systems is as yet insufficient. Reliability of adenovirus vector production is usually measured by means of determining from a number of independent adenovirus vectors produced, the number of independent adenovirus vectors capable of functionally expressing a nucleic acid of interest or an analogous method capable of determining an analogous value for instance in case said vector is not intended to express a nucleic acid of interest. Preferably at least 80%, more preferably at least 90% and most preferably at least 95% of the adenovirus vectors produced in a method of the invention are functional vectors and when present in said vector are capable of expressing said transgene and/or nucleic acid of interest. Reliability of adenovirus vector production is desired especially in applications where many different adenovirus vectors need to be produced in a relatively short time span. A reliable system for the production of adenovirus vectors then can significantly reduce the time and the costs involved. Preferably the efficiency of adenovirus vector production for an average vector is greater than 1 independent vector produced per 10^6 cells, more preferably the efficiency is more than 1 different vector produced 2×10^5 cells, most preferably the efficiency is more than 1 different vector produced 5×10^4 cells.

[0045] The applications of the disclosed inventions are outlined below and are illustrated in the Examples.

Use of the IG packaging constructs Diploid cells

[0046] The constructs, in particular pIG.E1A.E1B, will be used to transfect diploid human cells, such as Human Embryonic Retinoblasts (HER), Human Embryonic Kidney cells (HEK), and Human Embryonic Lung cells (HEL). Transfected cells will be selected for transformed phenotype (focus formation) and tested for their ability to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.IK. Such cell lines will be used for the generation and (large-scale) production of E1-deleted recombinant adenoviruses. Such cells, infected with recombinant adeno-

virus are also intended to be used *in vivo* as a local producer of recombinant adenovirus, e.g. for the treatment of solid tumors. 911 cells are used for the titration, generation and production of recombinant adenovirus vectors (Fallaux *et al.* (1996) *Hum. Gene Ther.* 7:215-222).

[0047] HER cells transfected with pLG.E1A.E1B has resulted in 7 independent clones (called PER cells). These clones are used for the production of E1 deleted (including nonoverlapping adenovirus vectors) or E1 defective recombinant adenovirus vectors and provide the basis for introduction of e.g. E2B or E2A constructs (e.g. ts125E2A see below). E4 etc., that will allow propagation of adenovirus vectors that have mutations in e.g. E2A or E4. In addition, diploid cells of other species that are permissive for human adenovirus such as the cotton rat (*Sigmodon hispidus*) (Pacini *et al.* (1984) *J. Infect. Dis.* 150:92-97), Syrian hamster (Morin *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 4626-4630) or chimpanzee (Levrero *et al.* (1991) *Gene* 101:195-202), will be immortalized with these constructs. Such cells, infected with recombinant adenovirus, are also intended to be used *in vivo* for the local production of recombinant adenovirus, e.g. for the treatment of solid tumors.

Established cells

[0048] The constructs, in particular pLG.E1A.NEO, can be used to transfect established cells, e.g. A549 (human bronchial carcinoma), KB (oral carcinoma), MRC-5 (human diploid lung cell line) or GLC cell lines (small cell lung cancer) de Leij *et al.* (1985) *Cancer Res.* 45:6024-6033; Postmus *et al.* (1988) *Eur. J. Clin. Oncol.* 24:753-763) and selected for NEO resistance. Individual colonies of resistant cells are isolated and tested for their capacity to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. When propagation of E1 deleted viruses on E1A containing cells is possible, such cells can be used for the generation and production of E1-deleted recombinant adenovirus. They are also used for the propagation of E1A deleted/E1B retained recombinant adenovirus.

[0049] Established cells can also be co-transfected with pLG.E1A.E1B and pLG.NEO (or another NEO containing expression vector). Clones resistant to G418 are tested for their ability to support propagation of E1 deleted recombinant adenovirus, such as IG.Ad.MLPI.TK and used for the generation and production of E1 deleted recombinant adenovirus and will be applied *in vivo* for local production of recombinant virus, as described for the diploid cells (see above).

[0050] All cell lines, including transformed diploid cell lines or NEO-resistant established lines, can be used as the basis for the generation of "next generation" packaging cell lines, that support propagation of E1-defective recombinant adenoviruses, that also carry deletions in other genes, such as E2A and E4. Moreover, they will provide the basis for the generation of minimal adenovirus vectors as disclosed herein.

E2 expressing cell lines

[0051] Packaging cells expressing E2A sequences are and will be used for the generation and (large scale) production of E2A-deleted recombinant adenovirus.

[0052] The newly generated human adenovirus packaging cell lines or cell lines derived from species permissive for human adenovirus (E2A or ts125E2A; E1A + E2A; E1A + E1B + E2A; E1A + E2A/ts 125; E1A + E1B + E2A/ts125) or non-permissive cell lines such as monkey cells (hrE2A or hr + ts125E2A; E1A + hrE2A; E1A + E1B + hrE2A; E1A + hrE2A/ts125; E1A + E1B + hrE2A/ts 125) are and will be used for the generation and (large scale) production of E2A deleted recombinant adenovirus vectors. In addition, they will be applied *in vivo* for local production of recombinant virus, as described for the diploid cells (see above).

Novel adenovirus vectors

[0053] The newly developed adenovirus vectors harboring an E1 deletion of nt. 459-3510 will be used for gene transfer purposes. These vectors may also be the basis for the development of further deleted adenovirus vectors that are mutated for e.g. E2A, E2B or E4. Such vectors will be generated e.g. on the newly developed packaging cell lines described above (see 1-3).

Minimal adenovirus packaging system

[0054] We disclose adenovirus packaging constructs (to be used for the packaging of minimal adenovirus vectors) may have the following characteristics:

- a) the packaging construct replicates
- b) the packaging construct cannot be packaged because the packaging signal is deleted.
- c) the packaging construct contains an internal hairpin-forming sequence (see section "Experimental; suggested hairpin" see Fig. 15)

d) because of the internal hairpin structure, the packaging construct is duplicated, that is the DNA of the packaging construct becomes twice as long as it was before transfection into the packaging cell (in our sample it duplicates from 35 kb to 70 kb). This duplication also prevents packaging. Note that this duplicated DNA molecule has ITR's at both termini (see e.g. Fig. 13)

e) this duplicated packaging molecule is able to replicate like a "normal adenovirus" DNA molecule

f) the duplication of the genome is a prerequisite for the production of sufficient levels of adenovirus proteins, required to package the minimal adenovirus vector

g) the packaging construct has no overlapping sequences with the minimal vector or cellular sequences that may lead to generation of RCA by homologous recombination

[0055] This packaging system will be used to produce minimal adenovirus vectors. The advantages of minimal adenovirus vectors e.g. for gene therapy of vaccination purposes, are well known (accommodation of up to 33 kb; gutting of all potentially toxic and immunogenic adenovirus genes)

[0056] Adenovirus vectors containing mutations in essential genes (including minimal adenovirus vectors) can also be propagated using this system.

Use of intracellular E2-expressing vectors

[0057] Minimal adenovirus vectors are generated using the helper functions provided in trans by packaging-deficient replicating helper molecules. The adenovirus-derived ITR sequences serve as origins of DNA replication in the presence of at least the E2-gene products. When the E2 gene products are expressed from genes in the vector genome (N.B. the gene(s) must be driven by an E1-independent promoter), the vector genome can replicate in the target cells. This will allow a significantly increased number of template molecules in the target cells, and, as a result an increased expression of the genes of interest encoded by the vector. This is of particular interest for approaches of gene therapy in cancer.

Applications of intracellular amplification of linear DNA fragments

[0058] A similar approach could also be taken if amplification of linear DNA fragments is desired. DNA fragments of known or unknown sequence could be amplified in cells containing the E2-gene products if at least one ITR sequence is located near or at its terminus. There are no apparent constraints on the size of the fragment. Even fragments much larger than the adenovirus genome (36 kb) should be amplified using this approach. It is thus possible to clone large fragments in mammalian cells without either shuttling the fragment into bacteria (such as *E. coli*) or use the polymerase chain reaction (P.C.R.). At the end stage of a productive adenovirus infection a single cell can contain over 100,000 copies of the viral genome. In the optimal situation, the linear DNA fragments can be amplified to similar levels. Thus, one should be able to extract more than 5 µg of DNA fragment per 10 million cells (for a 35-kbp fragment). This system can be used to express heterologous proteins (equivalent to the Simian Virus 40-based COS-cell system) for research or for therapeutic purposes. In addition, the system can be used to identify genes in large fragments of DNA. Random DNA fragments may be amplified (after addition of ITRS) and expressed during intracellular amplification. Election or, selection of those cells with the desired phenotype can be used to enrich the fragment of interest and to isolate the gene.

Gene correction vectors.

[0059] Gene therapy procedures may be divided into two different concepts, i.e., gene addition and gene replacement. Gene addition aims at introducing a therapeutic nucleic acid molecule into somatic cells of a patient, whereby expression of said therapeutic nucleic acid molecule is often under the control of a heterologous promoter and transcription termination signal. For example, when a patient suffers from an inherited disease, a functional copy of the defective nucleic acid molecule responsible for the disease phenotype is introduced into cells of the patient and, upon expression of said therapeutic nucleic acid molecule, the disease phenotype is corrected. Gene addition is, obviously, also used to accomplish expression of otherwise not expressed genes, such as, e.g., cytokine or suicide genes like HSV-TK to treat tumors. The gene replacement procedure aims at repairing at least one copy of a defective gene responsible for a disease phenotype. This can be achieved by introducing a functional version of a gene, or part thereof comprising the mutant site of that gene, in such a way that homologous recombination between said functional version and said defective gene occurs. Consequently, the defective gene or its mutant site is replaced by the functional version of that gene or part thereof. In this way, no nucleic acid material that is foreign to the species of which the patient is a member is expressed in the treated cells but at least one allele of the mutant gene is repaired. For the majority of the inherited diseases it is known that heterozygous carriers are not affected, or at least are affected to a lesser extent than a homozygous patient. Thus, gene replacement may be used for correction of inherited disorders. It is to be

understood that this also includes the repair of defective tumor suppressor genes.

[0060] For gene therapy purposes, it is preferable to retain the E3 region. E3 containing vectors will be superior to their E3 deleted counterparts because they are able to prevent or reduce host cells responses such as CTL lysis of adenovirus infected cells and cell lysis by TNF.

[0061] It will be understood that it may not be necessary to retain the whole E3 region in the vectors according to the invention, as long as the part retained still has the function of reducing the response of the host against infected cells. For example, expression of E3-14.7 kD alone may be sufficient to reduce early responses mediated by TNF (see Ginsberg, H.S. (1989) *Proc. Natl. Acad. Sci. USA* 86:3623-3627. Ginsberg, H.S. (1991) *Proc. Natl. Acad. Sci. USA* 88:1651-1655). These vectors are useful for gene therapy of inherited diseases such as cystic fibrosis, Duchenne molecular dystrophy, Hypercholesterolemia, blood clotting disorders (hemophilia) and the like. They also are useful in the therapy of acquired diseases, such as tumors, hepatitis, (auto) immune diseases, restenosis, rheumatoid and the like.

[0062] Advantages of gene replacement over gene addition include (1) expression regulation of the replacing gene is identical to the endogenous expression pattern, and (2) the procedure is safe, because no risk exists of insertion mutagenesis due to random integration.

A recombinant nucleic acid based system for the generation of adenovirus vectors.

[0063] In one aspect the invention provides a method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of combining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof. Said nucleic acid molecules together comprise at least a left ITR, a right ITR and an adenovirus encapsulation signal or functional parts, derivatives and/or analogues thereof. With overlapping sequences are meant sequences that comprise sufficient nucleic acid sequence similarity to allow homologous recombination. Sequence similarity is preferably more than 80% and more preferably more than 95%. With overlapping sequences are also meant complementary ends, of for instance a restriction enzyme site, wherein said nucleic acids are linked through hybridisation of said complementary ends. Said welding together may be performed through any means capable of physically linking two nucleic acid molecules. Preferably said welding together is performed through linking complementary ends resulting from restriction enzyme digestion of said nucleic acid molecules. More preferably said welding together is performed through homologous recombination of overlapping sequences in said nucleic acid.

[0064] In one embodiment the invention provides a method for generating an adenovirus vector comprising welding together through homologous recombination two nucleic acid molecules comprising partially overlapping sequences wherein said overlapping sequences allow essentially only one homologous recombination which leads to the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof. Very important in this embodiment of the invention is that said partially overlapping sequences allow essentially only homologous recombination leading to the generation of a functional adenovirus vector capable of being replicated and packaged into adenovirus particles in the presence of the required transacting functions. With essentially only one is meant that said overlapping sequences in each nucleic acid comprise essentially only one continuous sequence wherein homologous recombination leading to the generation of a functional adenovirus may occur. Within said continuous sequence the actual number of homologous recombination events may be higher than one. Non continuous overlapping sequences are not desired because they reduce the reliability of said method. Non continuous overlapping sequences are also not desired because they reduce the overall efficiency of said method, presumably due to the generation of undesired homologous recombination products.

[0065] A preferred embodiment of the invention provides a method of the invention wherein both of said nucleic acid molecules comprise only one adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof.

[0066] In one aspect of the invention one or both of said two nucleic acid molecules have undergone modifications prior to said welding together. Said modification may include the welding together of different nucleic acid molecules leading to the generation of one or both of said two nucleic acid molecules. In a preferred embodiment said different nucleic acids are welded together through homologous recombination of partially overlapping sequences.

[0067] In one aspect of the invention said welding together is performed in a cell or a functional part, derivative and/or analogue thereof. Preferably said cell is a mammalian cell. In a preferred embodiment said nucleic acid molecules are not capable of replicating in said mammalian cell prior to said welding together. Said replication is undesired since it reduces the reliability of the methods of the invention presumably through providing additional targets for undesired homologous recombination. Said replication is also not desired because it reduces the efficiency of the methods of the invention presumably because said replication competes for substrate or adenovirus transacting functions with the replication of said adenovirus vector.

[0068] In a preferred embodiment, one of said nucleic acid molecules is relatively small and the other is relatively large. This configuration is advantageous because it allows easy manipulation of said relatively small nucleic acid molecule allowing for example the generation of a large number of small nucleic acid molecules comprising different nucleic acid of interest for instance for the generation of an adenovirus vector library. Said configuration is also desired because it allows the production of a large batch of quality tested large nucleic acid molecule. The amplification of large nucleic acid molecules for instance in bacteria is difficult in terms of obtaining sufficient amounts of said large nucleic acid. The amplification of large nucleic acid molecules for instance in bacteria is also difficult to control because a small modification of said large nucleic acid is not easily detected. Moreover, for reasons not quite understood some large vectors are more stable in bacteria or yeasts than others. Said configuration however, allows the generation of a standard batch of a large nucleic acid molecule which can be thoroughly tested, for instance through generating a control adenovirus of which the efficiency and the reliability of production is known, and determining said parameters of a new batch of large nucleic acid molecule. Once validated said batch may be used for the generation of a large number of different adenovirus vectors through combining said large molecule with a large number of different small nucleic acid molecules. Said system therefore also allows for the selection and/or manipulation of vectors comprising a large nucleic acid molecule of the invention to allow a suitable yield of intact large nucleic acid.

[0069] In one embodiment of the invention at least one of said nucleic acid molecules comprises an adenovirus inverted terminal repeat which on one side is essentially free of other nucleic acid. An on one side essentially free adenovirus inverted terminal repeat is not essential for the generation of an adenovirus vector with a method or a means of the invention. However, an on one side essentially free adenovirus inverted terminal repeat enhances the efficiency of adenovirus vector production as compared to not essentially free inverted terminal repeat. With essentially free is meant that the outwardly directed end of said inverted terminal repeat is essentially free of additional nucleic acid bases. Some additional bases does not significantly affect the generation of adenovirus vectors particularly if said additional bases are not more than 50 bases, and preferably not more than 30 bases and more preferably not more than 10 bases. Preferably, both the left ITR and the right ITR are made essentially free of other nucleic acid on the outwardly directed side. Preferably, said adenovirus inverted terminal repeat is made essentially free of other nucleic acid on one side through restriction enzyme digestion of a restriction enzyme site present near said adenovirus inverted terminal repeat. Preferably, said restriction enzyme site is not present anywhere else in nucleic acid destined to be part of the adenovirus vector, in said nucleic acid molecule.

[0070] In one aspect the invention provides a method of the invention for the generation of an adenovirus vector wherein the nucleic acids present in said cell do not comprise sequence overlap leading to the formation of replication competent adenovirus. Other systems for the generation of adenovirus vectors do not sufficiently suppress the generation of replication competent adenovirus upon the generation and/or the propagation of adenovirus vectors. In one aspect of the present invention the generation of replication competent adenovirus is prevented through a system that prevents potential homologous recombination between for instance an E1-region and an adenovirus vector. Preventing the generation of replication competent adenovirus is very much desired when adenovirus vectors are to be used in a clinical setting. Preventing the generation of replication competent adenovirus increases the reliability of adenovirus production. Preventing the generation of replication competent adenovirus also increases the efficiency of adenovirus vector production.

[0071] One embodiment of the invention provides a method of the invention wherein the chromosomal nucleic acid in said cell comprises at least a functional part of an adenovirus E1-region, or a functional derivative and/or analogue thereof. Preferably said cell is a PER.C6 cell (ECACC deposit number 96022940) or a functional derivative and/or analogue thereof.

[0072] In another embodiment said nucleic acid in said cell further comprises a nucleic acid encoding an adenovirus E2-region and/or an adenovirus E4-region protein.

[0073] In another embodiment the invention provides a method or means for the generation of an adenovirus vector, wherein at least one of said nucleic acid molecules is linear. A linear molecule is not essential for adenovirus vector production however the efficiency of adenovirus vector production is increased compared to when circular or super-coiled molecules are used.

[0074] In one embodiment the invention provides a method according to the invention, wherein at least one of said molecules comprises adenovirus capsid proteins encoding nucleic acid derived from at least two different adenovirus serotypes. This embodiment of the invention is useful for the generation of for instance an adenovirus particle with a chimeric capsid comprising proteins from at least two different adenovirus serotypes. One advantageous feature of a chimeric capsid is the capacity to alter the tissue tropism of an adenovirus vector. The capsid of an adenovirus particle is among others a major determinant of whether a particle is capable of entering a certain cell type (tissue tropism) and by altering the capsid the tissue tropism of an adenovirus vector can be altered and designed to meet specific needs. Preferably said capsid comprises at least a tissue tropism determining part of a fiber protein of a subgroup B type adenovirus such as adenovirus 16 and at least one other capsid protein derived from a subgroup C type adenovirus such as adenovirus 5. Preferably said nucleic acid molecule comprising said adenovirus capsid protein encoding nucleic

acid is a large nucleic acid molecule, thus enabling the easy generation of a library of adenovirus vectors packaged into said chimeric capsid.

[0075] In another embodiment the invention provides a method of the invention wherein said welding together of said nucleic acid molecules leads to the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal, a nucleic acid encoding at least one adenovirus E1-region protein, at least one adenovirus E2-region encoded protein and/or at least one adenovirus E4-region encoded protein and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof and wherein at least one of said E1-region encoded proteins is under transcriptional control of a conditionally active promoter. With a conditionally active promoter is meant a promoter active in certain cell types and inactive in other cell types. The method of this embodiment is particularly useful for generation of a molecule capable of replicating in a cell provided that the conditionally active promoter is active in said cell. Such a molecule is useful for instance in vaccinations where very high expression of a transgene is required specifically in antigen presenting cells. When a vector of this embodiment is further provided with the capacity to express adenovirus capsid proteins said vector capable of replicating is also capable of being packaged in a cell provided that the conditionally active promoter is active in said cell. Thus forming a conditional replication incompetent adenovirus vector.

[0076] In another embodiment the invention provides a method of the invention wherein said physically linked nucleic acid comprises no other functional adenovirus nucleic acid than two inverted terminal repeats and a functional packaging signal or functional parts, derivatives and/or analogues thereof and wherein said physically linked nucleic acid is generated through welding together two nucleic acid molecules said molecules comprising partially overlapping sequences capable of combining with each other allowing the generation said physically linked nucleic acid. Said physically linked nucleic acid preferably further comprises a nucleic acid of interest. This setting is favorable as it allows the rapid generation of minimal adenovirus vectors through combining different relatively small nucleic acid molecules comprising different nucleic acids of interest with one tested and validated large nucleic acid molecule.

[0077] The invention further provides a method for generating an adenovirus vector with a deletion of the E2A gene comprising providing a cell with said nucleic acid molecules and growing said cell wherein a first nucleic acid comprises an adenovirus inverted terminal repeat and an encapsulation signal or functional parts, derivatives and/or analogues thereof, and a partially overlapping sequence allowing it to be welded together with a second nucleic acid molecule comprising an adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof, a deletion of at least part of the E2A gene and a partially overlapping sequence, wherein said cell is capable of expressing functional E2A, preferably a temperature sensitive E2A. Both or either one of said nucleic acids may further comprise a nucleic acid of interest operatively linked to a transcription unit like a promoter and poly-adenylation signal or functional parts, derivatives and/or analogues thereof. Welding together of said partially overlapping sequences may be achieved through any means provided capable of faithfully joining two strands of nucleic acid. Preferably said partially overlapping sequences are welded together through one homologous recombination. In a preferred embodiment said second nucleic acid is deleted of at least all E2A sequences that are present in said cell thus preventing homologous recombination that abolishes said deletion of E2A in said second nucleic acid or derivatives thereof resulting from said welding together.

[0078] The invention further provides a method for generating replication competent adenoviruses with a deletion in the E3 region comprising providing a cell with nucleic acid molecules and growing said cell wherein a first nucleic acid comprises an adenovirus inverted terminal repeat and encapsulation signal or functional parts, derivatives and/or analogues thereof, a conditionally functional E1 region, and a partially overlapping sequence allowing it to recombine with a second nucleic acid molecule comprising an adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof, a deletion in the E3 region and a partially overlapping sequence. Preferably said second nucleic acid contains a nucleic acid of interest in the E3 region. More preferably said nucleic acid of interest is operatively linked to the E3 promoter. Said nucleic acid of interest may be a suicide gene, a cytokine or a marker gene. Said second nucleic acid molecule may be generated in said cell by homologous recombination of two partially overlapping smaller nucleic acid molecules one of which comprises said deletion in the E3 region with or without a nucleic acid of interest and only one of said smaller nucleic acid molecules contains an adenoviral inverted terminal repeat preferably positioned on the end of one of said smaller nucleic acid molecules opposite of the partially overlapping sequence. The invention further provides a method to generate adenoviral vectors with a modification in at least one of the late genes comprising providing a cell with nucleic acid molecules and growing said cell wherein a first nucleic acid molecule comprises an adenovirus inverted terminal repeat and an encapsulation signal or functional parts, derivatives and/or analogues thereof, and a partially overlapping sequence allowing it to recombine with a second nucleic acid molecule comprising an adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof, a modification in at least one of the late genes and a partially overlapping sequence. Said modification in at least one of the late genes may comprise a modification in one of the capsid proteins, preferably penton or hexon or fiber or more preferably in more than one of the capsid proteins, more preferably in penton, hexon and fiber. Said modification may be a change in the nucleotide sequence resulting from mutagenesis, deletion, insertion or combinations thereof leading

to a functional change of said adenoviral vector in for example immunogenicity, infectivity or stability. Preferably said modification is a modification of said capsid genes generated by exchange of complete or part(s) of equivalent capsid genes from one or more different human or animal adenovirus serotypes, leading to a functional change of said adenoviral vector in for example immunogenicity, infectivity or stability. Said second nucleic acid molecule may be generated in said cell by homologous recombination of two partially overlapping smaller nucleic acid molecules at least one of which comprises said modification in one or more capsid genes whereby only one of said smaller nucleic acid molecules contains an adenoviral inverted terminal repeat or a functional part, derivative and/or analogue thereof, preferably positioned at the end of one of said smaller nucleic acid molecules opposite of said partially overlapping sequence.

[0079] The invention further provides a recombinant nucleic acid deposited under No. P97082122 at the ECACC.

[0080] The invention further provides a recombinant nucleic acid deposited under No. P97082119 at the ECACC.

[0081] The invention further provides a recombinant nucleic acid deposited under No. P97082117 at the ECACC.

[0082] The invention further provides a recombinant nucleic acid deposited under No. P97082114 at the ECACC.

[0083] The invention further provides a recombinant nucleic acid deposited under No. P97082120 at the ECACC.

[0084] The invention further provides a recombinant nucleic acid deposited under No. P97082121 at the ECACC.

[0085] The invention further provides a recombinant nucleic acid deposited under No. P97082116 at the ECACC.

[0086] The invention further provides a recombinant nucleic acid deposited under No. P97082115 at the ECACC.

[0087] The invention further provides a recombinant nucleic acid deposited under No. P97082118 at the ECACC.

[0088] The invention further provides a recombinant nucleic acid pWE/Ad.AfIII-EcoRI.

[0089] The invention further provides a recombinant nucleic acid comprising:

adenovirus derived nucleotides 1-454 and adenovirus nucleotides 3511-6095 shown in Figures 21 and 22.

[0090] The invention further provides a recombinant nucleic acid pAd5/CLIP.

[0091] The invention further provides a recombinant nucleic acid pAd5/L420-HSA.

[0092] The invention further provides a recombinant nucleic acid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔXbaI.

[0093] The invention further provides a recombinant nucleic acid according to the invention, wherein said nucleic acid further comprises a transgene.

[0094] The invention further provides a recombinant nucleic acid according to the invention, wherein said transgene is operatively linked to an E3 promoter.

[0095] The invention further provides a recombinant nucleic acid according to the invention, wherein said transgene comprises a suicide gene, a cytokine gene, or a reporter gene.

[0096] The invention further provides a recombinant nucleic acid according to the invention, wherein said suicide gene is an HSV-TK gene.

[0097] The invention further provides a recombinant nucleic acid according to the invention, wherein said transgene comprises a coding sequence selected from the group consisting of hIL-1 α , rat IL-3, and human IL-3.

[0098] The invention further provides a recombinant nucleic acid according to the invention, wherein said transgene comprises a coding sequence from a luciferase gene or a LacZ gene.

[0099] The invention further provides a recombinant nucleic acid according to the invention, wherein said transgene comprises a coding sequence from a human ceNOS gene.

[0100] The invention further provides a recombinant nucleic acid of the invention comprising a deletion in an E3 region of a recombinant nucleic acid.

[0101] The invention further provides a recombinant nucleic acid of the invention comprising a deletion in a gp19K region of a recombinant nucleic acid.

[0102] The invention further provides a recombinant nucleic acid comprising a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said recombinant nucleic acid has no functional adenovirus genes and no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which said recombinant nucleic acid is transferred. Preferably, said recombinant nucleic acid, further comprising a heterologous nucleotide sequence.

[0103] The invention further provides a recombinant nucleic acid pMV/L420-H.

[0104] The invention further provides a recombinant nucleic acid pMV/CMV-LacZ.

[0105] The invention further provides a recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises sufficient adenovirus sequences necessary for replication and capsid gene expression, wherein said nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus, and wherein said nucleotide sequence comprises no sequences which allow for homologous recombination leading to replication competent virus in a cell into which said recombinant nucleic acid is transferred.

[0106] The invention further provides a recombinant nucleic acid pWE/Ad.A5'.

[0107] The invention further provides a recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises sufficient adenovirus sequences necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid polymerase, wherein said nucleotide sequence comprises a deletion of one Inverted Terminal Repeat, the E1 region and the encapsulating signal of said adenovirus, and wherein said nucleic acid has no overlapping sequence which allow for homologous recombination leading to replication competent virus in a cell into which said nucleic acid is transferred. Preferably said molecule is pWE/AAV.Δ5'.

[0108] The invention further provides a recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenoviral sequences necessary for replication, wherein said nucleotide sequence comprises at least a deletion of the E1 region and encapsulating signal of said adenovirus, and wherein said nucleic acid has no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which said nucleic acid is transferred. Preferably, said nucleotide sequence further comprises a deletion of at least one of the Inverted Terminal Repeats of said adenovirus. Preferably, said sequence for adenovirus-independent replication comprises an SV40 origin of replication.

[0109] The invention further provides a recombinant nucleic acid pWE/Ad-H.

[0110] The invention further provides an adaptor plasmid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises in operable configuration at least one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination and the generation of a replication-defective, recombinant adenovirus genome, and wherein said adapter plasmid has no sequences which allow for homologous recombination leading to replication competent virus in a cell into which said adapter plasmid is transferred. Preferably said adapter plasmid comprises no E1 region sequences. Preferably said adapter plasmid further comprises a multiple cloning site. Also preferred is an adapter plasmid according to the invention, further comprising a nucleic acid inserted into said multiple cloning site.

[0111] In another embodiment the invention provides a method for the generation of recombinant adenovirus having an E1 deletion and a gp19K deletion, comprising the step of:

growing a cell comprising adenovirus complementing sequences transfected with

i) an adapter plasmid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises in operable configuration one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination leading to the generation of a replication-defective, recombinant adenovirus genome in a cell into which said adapter plasmid is transferred and having no E1 region sequences, and

ii) a recombinant nucleic acid comprising at least one second nucleotide sequence based on or derived from an adenovirus, wherein said at least one second nucleotide sequence comprises one Inverted Terminal Repeat and sufficient adenovirus sequences for replication and a partial overlap with said adapter plasmid, wherein said at least one second nucleotide sequence comprises a deletion of at least the E1 region, encapsulating signal and gp19K sequences; wherein said complementing sequences, said first nucleotide sequence and said at least one second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus having an E1 deletion and a gp19K deletion is generated. Preferably, said adapter plasmid further comprises a first heterologous nucleotide sequence inserted into said E1 region deletion and said recombinant nucleic acid further comprises a second heterologous nucleotide sequence inserted into said gp19K region.

[0112] In another embodiment the invention provides a method for the generation of recombinant adenovirus, comprising the step of:

growing a cell comprising adenovirus complementing sequences transfected with

i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and

ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises sufficient adenovirus sequences for replication, wherein said second nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated.

[0113] In another embodiment the invention provides a method for the generation of recombinant adenovirus, comprising the steps of:

growing a cell comprising adenovirus complementing sequences transfected with

i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and

ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenoviral sequences necessary for replication, wherein said second nucleotide sequence comprises at least a deletion of the E1 region and encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated. Preferably said cell comprises at least one nucleic acid molecule whereby said cell expresses SV40 Large T antigen proteins or functional fragments thereof. More preferably said second recombinant nucleic acid molecule is replicated.

[0114] In one embodiment the invention provides a replication defective adenovirus comprising:

a genome based on or derived from an adenovirus, wherein said genome comprises at least a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof and wherein said genome comprises no functional adenovirus genes and has no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which said replication defective adenovirus is transferred. Preferably said replication defective adenovirus further comprises one or more expression cassettes. Preferably, said expression cassette comprises a gene functionally linked to transcription regulatory sequences.

[0115] In one embodiment said replication defective adenovirus further comprises one or more non-adenoviral nucleic acid sequences. Preferably said one or more non-adenoviral nucleic acid sequences are inserted in the E1 region or in the E3 region gp19K gene.

[0116] In another aspect the invention provides a non-human cell comprising a genome of a replication defective adenovirus according to the invention. Preferably said cell is a mammalian cell.

[0117] In another aspect the invention provides a method for transducing a cell, comprising the step of:

contacting said cell with a replication defective adenovirus according to the invention under conditions whereby said cell is transduced.

[0118] In another aspect the invention provides a non-human cell produced according to a method of the invention, preferably said cell is a mammalian cell.

[0119] In one embodiment the invention provides a method for generating recombinant adenovirus comprising the step of:

growing a cell comprising adenovirus complementing sequences and

i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and,

ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises at least all adenovirus sequences, or functional fragments or derivatives thereof necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid polymerase, wherein said second nucleotide sequence comprises a deletion of one Inverted Terminal Repeat, the E1 region and the encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated.

[0120] In another aspect the invention provides a cell comprising a recombinant nucleic acid and/or an adapter plasmid according to the invention

[0121] In yet a further aspect the invention provides a method for the replacement of a defective gene in a host cell genome comprising the step of:

growing said host cell with a recombinant nucleic acid molecule derived from a replication defective adenovirus comprising a functional version or part thereof of said defective gene under conditions whereby at least one allele of said defective gene in said host cell genome is replaced.

[0122] In one embodiment the invention provides a method for transducing a cell according to the invention, wherein said replication defective adenovirus expresses no adenoviral genes. Preferably said defective gene is a defective tumor suppressor gene.

[0123] The invention further provides an isolated cell comprising a genome of a replication-defective adenovirus according to the invention. Preferably said cell is a human cell.

[0124] The invention further provides a recombinant nucleic acid according to the invention, wherein said deletion in the E3 region is replaced with a transgene.

[0125] The invention further provides a recombinant nucleic acid according to the invention, wherein said deletion in the gp19K region is replaced with a transgene.

[0126] The invention further provides a method according to the invention, wherein said at least one second nucleotide sequence comprises a first and second molecule wherein said first molecule has said partial overlap with said adapter plasmid at the 3' end, and said second molecule comprises said Inverted Terminal Repeat and region including deletion of said gp19K sequences.

[0127] The invention further provides a replication-defective adenovirus comprising: a genome based on or derived from an adenovirus, wherein said genome comprises a first deletion, in an E1 region and a second deletion in a gp19K region. Preferably transcription of said transgene is directed by an E3 promoter.

[0128] The invention further provides an isolated cell comprising:

a recombinant nucleic acid and/or an adapter plasmid according to the invention. Preferably said cell is a human cell.

[0129] The following examples are presented as illustrations, not limitations.

EXAMPLES

Example I

Generation of cell lines able to transcomplement E1 defective recombinant adenovirus vectors

1. 911 cell line

[0130] A cell line that harbors E1 sequences of adenovirus type 5, able to trans-complement E1 deleted recombinant adenovirus has been generated (Fallaux *et al*, (1996) *Hum. Gene Ther.* 7:215-222). This cell line was obtained by transfection of human diploid human embryonic retinoblasts (HER) with pAd5XhoIC, that contains nt. 80-5788 of Ad 5; one of the resulting transformants was designated 911. This cell line has been shown to be useful in the propagation of E1 defective recombinant adenovirus. It was found to be superior to the 293 cells. Unlike 293 cells, 911 cells lack a fully transformed phenotype, which most likely is the cause of performing better as adenovirus packaging line:

plaque assays can be performed faster (4-5 days instead of 8-14 days on 293) monolayers of 911 cells survive better under agar overlay as required for plaque assays higher amplification of E1-deleted vectors.

[0131] In addition, unlike 293 cells that were transfected with sheared adenoviral DNA, 911 cells were transfected using a defined construct. Transfection efficiencies of 911 cells are comparable to those of 293.

New packaging constructs

Source of adenovirus sequences

[0132] Adenovirus sequences are derived either from pAd5.SalB, containing nt. 80-9460 of human adenovirus type 5 (Bernards *et al*, (1983) *Virology* 127:45-53) or from wild-type Ad5 DNA. pAd5.SalB was digested with *SalI* and *XhoI* and the large fragment was religated and this new clone was named pAd5.X/S. The pTN construct (constructed by Dr. R. Vogels, IntroGene, The Netherlands) was used as a source for the human PGK promoter and the NEO gene.

Human PGK Promoter and NEO^R gene

[0133] Transcription of E1A sequences in the new packaging constructs is driven by the human PGK promoter (Michelson *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:472-476). Singer-Sam *et al.* (1984) *Gene* 32:409-417), derived from plasmid pTN (gift of R. Vogels), which uses pUC 119 (Vieira *et al.* (1987) pp. 3-11: *Methods in Enzymology*, Acad. Press Inc.) as a backbone. This plasmid was also used as a source for the NEO gene fused to the Hepatitis B Virus (HBV) poly-adenylation signal.

Fusion of PGK promoter to E1 genes (Fig. 1)

[0134] In order to replace the E1 sequences of Ad5 (ITR, origin of replication and packaging signal) by heterologous sequences we have amplified E1 sequences (nt.459 to nt.960) of Ad5 by PCR, using primers Ea1 and Ea2 (see Table 1). The resulting PCR product was digested with *Cla*I and ligated into Bluescript (Stratagene) predigested with *Cla*I and *Eco*RV resulting in construct pBS.PCRI.

[0135] Vector pTN was digested with restriction enzymes *Eco*RI (partially) and *Sal*I, and the DNA fragment containing the PGK promoter sequences was ligated into pBS.PCRI digested with *Sal*I and *Eco*RI. The resulting construct pBS.PGK.PCRI contains the human PGK promoter operatively linked to Ad5 E1 sequences from nt.459 to nt.916.

Construction of pG.E1A.E1B (Fig. 2)

[0136] pG.E1A.E1B.X contains the E1A and E1B coding sequences under the direction of the PGK promoter. As Ad5 sequences from nt.459 to nt.5788 are present in this construct, also pIX protein of adenovirus is encoded by this plasmid. pG.E1A.E1B.X was made by replacing the *Sal*I-*Bsp*EI fragment of pAT-X/S by the corresponding fragment from pBS.PGK.PCRI (containing the PGK promoter linked to E1A sequences).

Construction of pG.E1A.NEO (Fig. 3)

[0137] In order to introduce the complete E1B promoter and to fuse this promoter in such a way that the AUG codon of E1B 21 kD exactly functions as the AUG codon of NEO^R, the E1B promoter was amplified using primers Ea3 and Ep2, where primer Ep2 introduces a *Nco*I site in the PCR fragment. The resulting PCR fragment, named PCRII, was digested with *Hpa*I and *Nco*I and ligated into pAT-X/S, which was predigested with *Hpa*I and with *Nco*I. The resulting plasmid was designated pAT-X/S-PCR2. The *Nco*I-*Stu*I fragment of pTN, containing the NEO gene and part of the Hepatitis B Virus (HBV) poly-adenylation signal, was cloned into pAT-X/SPCR2 which had been digested with *Nco*I and *Nru*I. The resulting construct was pAT.PCR2.NEO. The poly-adenylation signal was completed by replacing the *Sal*I-*Sal*I fragment of pAT.PCR2.NEO with the corresponding fragment of pTN, resulting in pAT.PCR2.NEO.p(A). The *Sal*I-*Xba*I of pAT.PCR2.NEO.p(A) was replaced with the corresponding fragment of pG.E1A.E1B-X, containing the PGK promoter linked to E1A genes. The resulting construct was named pG.E1A.NEO, and thus contains Ad5 E1 sequences (nt.459 to nt.1713) under the control of the human PGK promoter.

Construction of pG.E1A.E1B (Fig. 4)

[0138] pG.E1A.E1B contains nt.459 to nt.3510 of Ad5, that encode the E1A and E1B proteins. The E1B sequences are terminated at the splice acceptor at nt.3511. No pIX sequences are present in this construct.

[0139] pG.E1A.E1B was made as follows: The sequences encoding the N-terminal amino acids of E1B 55 kD were amplified using primers Eb1 and Eb2 which introduces a *Xho*I site. The resulting PCR fragment was digested with *Bgl*II and cloned into *Bgl*III/*Nru*I of pAT-X/S, thereby obtaining pAT-PCR3. The HBV poly (A) sequences of pG.E1A.NEO were introduced downstream of the E1B sequences of pAT-PCR3 by exchange of the *Xba*I-*Sal*I fragment of pG.E1A.NEO and the *Xba*I-*Xho*I fragment of pAT-PCR3.

Construction of pG.NEO (Fig. 5)

[0140] This construct is of use when established cells are transfected with E1A.E1B constructs and NEO selection is required. Because NEO expression is directed by the E1B promoter, NEO resistant cells are expected to co-express E1A, which also is advantageous for maintaining high levels of expression of E1A during long-term culture of the cells. pG.NEO was generated by cloning the *Hpa*I-*Sal*I fragment of pG.E1A.NEO, containing the NEO gene under the control of the Ad5 E1B promoter, into pBS digested with *Eco*RV and *Sal*I.

Testing of constructs

[0141] The integrity of the constructs pIG.E1A.NEO, pIG.E1A.E1B.X and pIG.E1A.E1B was assessed by restriction enzyme mapping; furthermore, parts of the constructs that were obtained by PCR analysis were confirmed by sequence analysis. No changes in the nucleotide sequence were found.

[0142] The constructs were transfected into primary BRK (Baby Rat Kidney) cells and tested for their ability to immortalize (pIG.E1A.NEO) or fully transform (pAd5.XhoI.C. pIG.E1A.E1B.X and pIG.E1A.E1B) these cells. Kidneys of 6-day old WAG-Rij rats were isolated, homogenized and trypsinized. Subconfluent dishes (diameter 5 cm) of the BRK cell cultures were transfected with 1 or 5 µg of pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG.E1A.E1B.X, pAd5.XhoI.C. or with pIG.E1A.NEO together with PDC26 (Elsen *et al.*, (1983) *Virology* 128:377-390), carrying the Ad5.E1B gene under control of the SV40 early promoter. Three weeks post-transfection, when foci were visible, the dishes were fixed, Giemsa stained and the foci counted.

[0143] An overview of the generated adenovirus packaging constructs, and their ability to transform BRK, is presented in Fig. 6. The results indicate that the constructs pIG.E1A.E1B and pIG.E1A.E1B.X are able to transform BRK cells in a dose-dependent manner. The efficiency of transformation is similar for both constructs and is comparable to what was found with the construct that was used to make 911 cells, namely pAd5.XhoI.C.

[0144] As expected, pIG.E1A.NEO was hardly able to immortalize BRK. However, co-transfection of an E1B expression construct (PDC26) did result in a significant increase of the number of transformants (18 versus 1), indicating that the E1A encoded by pIG.E1A.NEO is functional. We conclude therefore, that the newly generated packaging constructs are suitable for the generation of new adenovirus packaging lines.

Generation of cell lines with new packaging constructs cell lines and cell culture

[0145] Human A549 bronchial carcinoma cells (Shapiro *et al.*, (1973) *Biochem. Biophys. Acta* 530:197-207), human embryonic retinoblasts (HER), Ad5-E1-transformed human embryonic kidney (HEK) cells (293; Graham *et al.*, (1977) *J. Gen. Virol.* 36:59-72) and Ad5-transformed HER cells (911; Fallaux *et al.*, (1996). *Hum. Gene Ther.* 7:215-222) and PER cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics in a 5% CO₂ atmosphere at 37°C. Cell culture media, reagents and sera were purchased from Gibco Laboratories (Grand Island, NY). Culture plastics were purchased from Greiner (Nürtingen, Germany) and Corning (Cooring, NY).

Viruses and virus techniques

[0146] The construction of recombinant adenoviral vectors IG.Ad.MLP.nls.lacZ, IG.Ad.NILP.luc, IG.Ad.MLP.TK and IG.Ad.CW.TK is described in detail in patent application EP 95202213. The recombinant adenoviral vector IG.Ad.MIP.nls.lacZ contains the *E. coli* lacZ gene, encoding β-galactosidase, under control of the Ad2 major late promoter (MLP), IG.Ad.MLP.luc contains the firefly luciferase gene drive by the Ad2 MLP, and adenoviral vectors IG.Ad.NLP.TK and IG.Ad.CMV.TK contain the Herpes Simplex Virus thymidine kinase (TK) gene under the control of the Ad2 MLP and the Cytomegalovirus (CMV) enhancer/promoter, respectively.

Transfections

[0147] All transfections were performed by calcium-phosphate precipitation DNA (Graham *et al.*, (1973) *Virology* 52:456-467) with the GIBCO Calcium Phosphate Transfection System (GIBCO BRL Life Technologies, Inc., Gaithersburg, USA), according to the manufacturer's protocol.

Western blotting

[0148] Subconfluent cultures of exponentially growing 293, 911 and Ad5-E1-transformed A549 and PER cells were washed with PBS and scraped in Fos-RIPA buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 1% NA-DCC, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), 0.5 mM trypsin inhibitor, 50 mM NaF and 1 mM sodium vanadate). After 10 min. at room temperature, lysates were cleared by centrifugation. Protein concentrations were measured with the BioRad protein assay kit and 25 µg total cellular protein was loaded on a 12.5% SDS-PAA gel. After electrophoresis, proteins were transferred to nitrocellulose (1h at 300 mA). Prestained standards (Sigma, USA) were run in parallel. Filters were blocked with 1% bovine serum albumin (BSA) in TBST (10 mM Tris, pH 8.15 mM NaCl, and 0.05% Tween-20) for 1 hour. First antibodies were the mouse monoclonal anti-Ad5-E1B-55-kDa antibody A1C6 (Zantera *et al.*, unpublished), the rat monoclonal anti-Ad5-E1B-221-kDa antibody C1G11 (Zantera *et al.*, (1985) *Virology* 142:44-58). The second antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Promega).

Signals were visualized by enhanced chemoluminescence (Amersham Corp. UK)

Southern blot analysis

[0149] High molecular weight DNA was isolated and 10 µg was digested to completion and fractionated on a 0.7% agarose gel. Southern blot transfer to Hybond N+ (Amersham, UK) was performed with a 0.4 M NaOH, 0.6 M NaCl transfer solution (Church and Gilbert, 1984). Hybridization was performed with a 2463-nt *SspI-HindIII* fragment from pAd5.SalB (Bernards et al, (1983) *Virology* 127:45-53). This fragment consists of Ad5 bp. 342-2805. The fragment was radiolabeled with α , α -³²P-dCTP with the use of random hexanucleotide primers and Klenow DNA polymerase. The southern blots were exposed to a Kodak XAR-5 film at -80°C and to a Phosphor-Imager screen which was analyzed by B&L systems Molecular Dynamics Software.

A549

[0150] Ad5-E1-transformed A549 human bronchial carcinoma cell lines were generated by transfection with pIG.E1A.NEO and selection for G418 resistance. Thirty-one G418 resistant clones were established. Co-transfection of pIG.E1A.E1B with pIG.NEO yielded seven G418 resistant cell lines.

PER

[0151] Ad5-E1-transformed human embryonic retina (HER) cells were generated by transfection of primary HER cells with plasmid pIG.E1A.E1B. Transformed cell lines were established from well-separated foci. We were able to establish seven clonal cell lines, which we called PER.C1, PER.C3, PER.C4, PER.C5, PER.C6, PER.C8 and PER.C9. One of the PER clones, namely PER.C6, has been deposited at the ECACC under number 96022940.

Expression of Ad5 E1A and E1B genes in transformed A549 and PER cells

[0152] Expression of the Ad5 E1A and the 55-kDa and 21 kDa E1B proteins in the established A549 and PER cells was studied by means of Western blotting, with the use of monoclonal antibodies (mAb). mAb M73 recognizes the E1A products, whereas Mabs A1C6 and C1G11 are directed against the 55-kDa and 21 kDa E1B proteins, respectively. The antibodies did not recognize proteins in extracts from the parental A549 or the primary HER cells (data not shown). None of the A549 clones that were generated by co-transfection of pIG.NEO and pIG.E1A.E1B expressed detectable levels of E1A or E1B proteins (not shown). Some of the A549 clones that were generated by transfection with pIG.E1A.NEO expressed the Ad5 E1A proteins (Fig. 7), but the levels were much lower than those detected in protein lysates from 293 cells. The steady state E1A levels detected in protein extracts from PER cells were much higher than those detected in extracts from A549-derived cells. All PER cell lines expressed similar levels of E1A proteins (Fig. 7). The expression of the E1B proteins, particularly in the case of E1B 55 kDa, was more variable. Compared to 911 and 293, the majority of the PER clones express high levels of E1B 55 kDa and 2 kDa. The steady state level of E1B 21 kDa was the highest in PER.C3. None of the PER clones lost expression of the Ad5 E1 genes upon serial passage of the cells (not shown). We found that the level of E1 expression in PER cells remained stable for at least 100 population doublings. We decided to characterize the PER clones in more detail.

Southern analysis of PER clones

[0153] To study the arrangement of the Ad5-E1 encoding sequences in the PER clones we performed Southern analyses. Cellular DNA was extracted from all PER clones, and from 293 and 911 cells. The DNA was digested with *HindIII*, which cuts once in the Ad5 E1 region. Southern hybridization on *HindIII*-digested DNA, using a radiolabeled Ad5-E1-specific probe revealed the presence of several integrated copies of pIG.E1A.E1B in the genome of the PER clones. Figure 8 shows the distribution pattern of E1 sequences in the high molecular weight DNA of the different PER cell lines. The copies are concentrated in a single band, which suggests that they are integrated as tandem repeats. In the case of PER.C3, C5, C6 and C9 we found additional hybridizing bands of low molecular weight that indicate the presence of truncated copies of pIG.E1A.E1B. The number of copies was determined with the use of a Phosphor-Imager. We estimated that PER.C1, C3, C4, C5, C6, C8 and C9 contain 2, 88, 5, 4, 5, 5, and 3 copies of the Ad5 E1 coding region, respectively, and that 911 and 293 cells contain 1 and 4 copies of the Ad5 E1 sequences, respectively.

Transfection efficiency

[0154] Recombinant adenovectors are generated by co-transfection of adapter plasmids and the large *ClaI* fragment

of Ad5 into 293 cells (EP application 95202213). The recombinant virus DNA is formed by homologous recombination between the homologous viral sequences that are present in the plasmid and the adenovirus DNA. The efficacy of this method, as well as that of alternative strategies, is highly dependent on the transfectability of the helper cells. Therefore, we compared the transfection efficiencies of some of the PER clones with 911 cells, using the *E. coli* β -galactosidase-encoding LacZ gene as a reporter (Fig. 9)

Production of recombinant adenovirus

[0155] Yields of recombinant adenovirus obtained after inoculation of 293, 911, PER C3, PER.C5 and PER.C6 with different adenovirus vectors are presented in Table II.

[0156] The results indicate that the recombinant adenovirus vector yields obtained with PER cells are at least as high as those obtained with the existing cell lines. In addition, the yields of the novel adenovirus vector IG.Ad.MLPI.TK are similar or higher than the yields obtained for the other viral vectors on all cell lines tested.

Generation of new adenovirus vectors (Fig. 10)

[0157] The recombinant adenovirus vectors used (see patent application EP 95202213) are deleted for E1 sequences from 459 to nt. 3328. As construct pE1A.E1B contains Ad5 sequences 459 to nt. 3510 there is a sequence overlap of 183 nt. between E1B sequences in the packaging construct pIG.E1A.E1B and recombinant adenoviruses, such as for example IG.Ad.MLP.TK. The overlapping sequences were deleted from the new adenovirus vectors. In addition, non-coding sequences derived from LacZ, that are present in the original constructs, were deleted as well. This was achieved (see Fig. 10) by PCR amplification of the SV40 poly (A) sequences from pMLP.TK using primers SV40-1 (introduces a *Bam*HI site) and SV40-2 (introduces a *Bgl*II site). In addition, Ad5 sequences present in this construct were amplified from nt. 2496 (Ad5-1, introduces a *Bgl*II site) to nt. 2779 (Ad5-2). Both PCR fragments were digested with *Bgl*II and were ligated. The ligation product was PCR amplified using primers SV40-1 and Ad5-2. The PCR product obtained was cut with *Bam*HI and *A*II and was ligated into pMLP.TK predigested with the same enzymes. The resulting construct, named pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt. 459 to nt. 3510.

Packaging system

[0158] The combination of the new packaging construct pIG.E1A.E1B and the recombinant adenovirus pMLPI.TK, which do not have any sequence overlap, are presented in Fig. 11. In this figure also the original situation is presented, where the sequence overlap is indicated. The absence of overlapping sequences between pIG.E1A.E1B and pMLPI.TK (Fig. 11a) excludes the possibility of homologous recombination between the packaging construct and the recombinant virus, and is therefore a significant improvement for production of recombinant adenovirus as compared to the original situation.

[0159] In Fig. 11b the situation is depicted for pIG.E1A.NEO and IG.Ad.MLPI.TK. pIG.E1A.NEO when transfected into established cells, is expected to be sufficient to support propagation of E1-deleted recombinant adenovirus. This combination does not have any sequence overlap, preventing generation of RCA by homologous recombination. In addition, this convenient packaging system allows the propagation of recombinant adenoviruses that are deleted just for E1A sequences and not for E1B sequences.

[0160] Recombinant adenoviruses expressing E1B in the absence of E1A are attractive, as the E1B protein, in particular E1B 19 kD, is able to prevent infected human cells from lysis by Tumor Necrosis Factor (TNF) Gooding *et al*, (1991) *J. Virol.* 65:3083-3094).

Generation of recombinant adenovirus derived from pMLPI.TK

[0161] Recombinant adenovirus was generated by co-transfection of 293 cells with *Sa*I linearized pMLPI.TK DNA and *Cl*aI linearized Ad5 wt DNA. The procedure is schematically represented in Fig. 12.

Example 2**Plasmid-based system for rapid RCA-free generation of recombinant adenoviral vectors****A. Construction of adenovirus clones****pBr/Ad.Bam-rlTR (ECACC deposit P970821212)**

[0162] In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with *Bam*HI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with *Eco*RV and *Bam*HI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LNP agarose gel (SeaPlaque GTG). After transformation into competent *E. coli* DH5 α (Life Techn.) and analysis of ampicillin resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the *Bam*HI site in Ad5 to the right ITR. Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

pBr/Ad.Sal-rlTR (ECACC deposit P97082119)

[0163] pBr/Ad.Bam-rlTR was digested with *Bam*HI and *Sal*I. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb *Sal*/BamHI fragment obtained from wt Ad5 DNA and purified with the GeneClean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rlTR contains adeno type 5 sequences from the *Sal*I site at bp 16746 up to and including the rlTR (missing the most 3'G residue).

pBr/Ad.Cla-Bam (ECACC deposit P97082117)

[0164] wt Adeno type 5 DNA was digested with *Clal* and *Bam*HI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by GeneClean. Both fragments were ligated and transformed into competent DH5 α . The resulting clone pBr/Ad.Cla-Bam was analyzed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

pBr/Ad.AflI-Bam (ECACC deposit P97082114)

[0165] Clone pBr/Ad.Cla-Bam was linearized with *Eco*RI (in pBr322) and partially digested with *Afl*I. After heat inactivation of *Afl*I for 20 minutes at 65°C, the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a *Pac*I site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess *Pac*I enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5 α . One clone that was found to contain the *Pac*I site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the *Pac*I linker in the (lost) *Afl*I site.

pBr/Ad.Bam-rlTRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rlTR#8 (ECACC deposit P97082121)

[0166] To allow insertion of a *Pac*I site near the ITR of Ad5 in clone pBr/Ad.Bam-rlTR about 190 nucleotides were removed between the *Clal* site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rlTR was digested with *Clal* and treated with nuclease *Ba*31 for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the *Clal* site), using identical buffers and conditions. *Ba*31 enzyme was inactivated by incubation at 75°C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with *Sal*I, satisfactory degradation (~150 bp) was observed in the samples treated for 10 minutes or 15 minutes. The 10 minutes or 15 minutes treated pBr/Ad.Bam-rlTR samples were then ligated to the above described blunted *Pac*I linkers (see

pBr/Ad.AfIII-Bam). Ligations were purified by precipitation, digested with excess *PacI* and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5a and colonies analyzed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit Pharmacia Biotech). Two clones were found with the *PacI* linker inserted just downstream of the RITR. After digestion with *PacI*, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

pWE/Ad.AfIII-rITR (ECACC deposit P97082116)

[0167] Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique *PacI* site was inserted in the *EcoRI* sites of pWE15 creating pWE.pac. To this end, the double stranded *PacI* oligo as described for pBr/Ad.AfIII-BamHI was used but now with its *EcoRI* protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with *PacI*, pBr/AfIII-Bam digested with *PacI* and *BamHI* and pBr/Ad.Bam-rITR#2 digested with *BamHI* and *PacI*. These fragments were listed together and packaged using λ phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AfIII-rITR contains all adenovirus type 5 sequences, from bp 3534 (*AflI* site) up to and including the right ITR (missing the most 3' G residue).

pBr/Ad.IITR-Sal(9.4) (ECACC deposit P97082115)

[0168] Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with *SalI*. Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with *EcoRV* and *SalI* and treated with phosphatase (Life Technologies). The vector fragment was isolated using the GeneClean method (BIO 101, Inc.) and ligated to the Ad5 *SalI* fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to the *SalI* site at bp 9462.

pBr/Ad.IITR-Sal(16.7) (ECACC deposit P97082118)

[0169] pBr/Ad.IITR-Sal(9.4) is digested with *SalI* and dephosphorylated (TSAP, Life Technologies). To extend this clone up to the third *SalI* site in Ad5, pBr/Ad.Cla-Bam was linearized with *BamHI* and partially digested with *SalI*. A 7.3 kb *SalI* fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the *SalI* digested pBr/Ad.IITR-Sal(9.4) vector fragment.

pWE/Ad.AfIII-EcoRI

[0170] pWE.pac was digested with *Clal* and the 5' protruding ends were filled in using Klenow enzyme. The DNA was then digested with *PacI* and isolated from agarose gel. pWE/AfIII-rITR was digested with *EcoRI* and after treatment with Klenow enzyme digested with *PacI*. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the *Clal*-digested and blunted pWE.pac vector using the Ligation Express[™] kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AfIII-EcoRI contains Ad5 sequences from bp 3534-27336.

B. Construction of new adapter plasmids

[0171] The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (Fig. 10) is an example of an adapter plasmid designed for use according to the invention in combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.

[0172] First, a PCR fragment was generated from pZipΔMo+PyF101(N⁻) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to the manufacturer's protocol with the following temperature cycles: once 5 minutes at 95°C; 3 minutes at 55°C; and 1 minute at 72°C, and 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, followed by once 10 minutes at 72°C. The PCR product was then digested with *BamHI* and ligated into a pMLP10 (Levrero *et al.*, (1991) *Gene* 101: 195-202) vector digested with *PvuII* and *BamHI*, thereby generating vector pLTR10. This vector contains adenoviral sequences

from bp 1 up to bp 454 followed by a promoter which includes part of the Mo-MuLV LTR in which the wild-type enhancer sequences are replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420.

[0173] Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with *Bst*BI followed by Klenow treatment and digestion with *Nco*I. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay *et al.*, (1990) *J. Immunol.* 145:1952-1959) using the following primers. HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified fragment was subcloned in a shuttle vector using the *Nco*I and *Bgl*II sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication was then excised as a *Nco*I (sticky)-*Sal*I (blunt) fragment and cloned into the 3.5 kb *Nco*I (sticky)/*Bst*BI (blunt) fragment from pLTR10, resulting in pLTR-HSA10.

[0174] Finally, pLTR-HSA10 was digested with *Eco*RI and *Bam*HI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLP1.TK digested with the same enzymes, thereby replacing the promoter and the gene sequences. This resulted in the new adapter plasmid pAd/L420-HAS (Fig. 21) that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. *Sna*BI and *Avr*II can be combined with *Hpa*I, *Nhe*I, *Kpn*I, *Hind*III to exchange promoter sequences, while the latter sites can be combined with the *Cla*I or *Bam*HI sites 3' from the HSA coding region to replace genes in this construct.

[0175] Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with *Avr*II and *Bgl*II, followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with *Hha*I and *Avr*II followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP (Fig. 22).

C. Generation of recombinant adenoviruses

E1-deleted recombinant adenoviruses with wt E3 sequences

[0176] To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs were prepared: an adapter construct containing the expression cassette with the gene of interest linearized with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences; and a complementing adenoviral genome construct pWE/Ad.AfIII-rITR digested with *Pac*I.

[0177] These two DNA molecules are further purified by phenol/chloroform extraction and ETOH precipitation. Co-transfection of these plasmids into an adenovirus packaging cell line, preferably a cell line according to the invention, generates recombinant replication deficient adenoviruses by a one-step homologous recombination between the adapter and the complementing construct (Fig. 23). Alternatively, instead of pWE/Ad.AfIII-rITR other fragments can be used, e.g. pBr/Ad.Cla-Bam digested with *Eco*RI and *Bam*HI or pBr/Ad.AfIII-BamHI digested with *Pac*I and *Bam*HI can be combined with pBr/Ad.Sal-rITR digested with *Sal*I.

[0178] Recombinant adenovirus can be produced following introduction of said plasmids in said cell. It is to be understood that those skilled in the art may use other combinations of adapter and complementing plasmids without departing from the present invention.

[0179] A general protocol as outlined below and meant as a non-limiting example of the present invention has been performed to produce several recombinant adenoviruses using various adapter plasmids and the Ad.AfIII-rITR fragment. Adenovirus packaging cells (PER.C6) were seeded in ~25 cm² flasks and the next day when they were at ~80% confluency, were transfected with a mixture of DNA and lipofectamine agent (Life Techn.) as described by the manufacturer. Routinely, 40 µl lipofectamine, 4 µg adapter plasmid and 4 µg of the complementing adenovirus genome fragment AfIII-rITR (or 2 µg of all three plasmids for the double homologous recombination) were used. Under these conditions transient transfection efficiencies of ~50% (48 hrs post transfection) were obtained as determined with control transfections using a pAd/CMV-LacZ adapter. Two days later, cells were passaged to ~80 cm² flasks and further cultured. Approximately five (for the single homologous recombination) to eleven days (for the double homologous recombination) later a cytopathic effect (CPE) was seen, indicating that functional adenovirus has formed. Cells and medium are harvested upon full CPE and recombinant virus is released by freeze-thawing. An extra amplification step in a 80 cm² flask was routinely performed to increase the yield since at the initial stage the titers were found to be variable despite the occurrence of full CPE. After amplification, viruses were harvested and plaque purified on PER.C6 cells. Individual plaques were tested for viruses with active transgenes.

[0180] Four different recombinant adenoviruses, containing the human interleukin-3 gene (see Figure 1, W066/04691), the human endothelial nitric oxide gene (Janssens *et al.*, (1992) *J Biol Chem.* 267:14519-14522), the Tc1A transposase gene (Vos *et al.*, (1993) *Genes Dev.* 7:1244-1253), or the bacterial LacZ gene (Kalderon *et al.*, (1984) *Cell* 39:499-509, have been produced using this protocol. In all cases, functional adenovirus was formed and all isolated

E1-deleted recombinant adenoviruses with modifications in the E3 or E4 regions

[0181] Besides replacements in the E1 region it is possible to delete the E3 region or replace part of the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of a recombinant virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum packagable size (approximately 105% of wt genome length). This can be done, for example, by deleting part of the E3 region in the pBr/Ad Bam-rITR clone by digestion with *Xba*I and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This leaves all other coding regions intact, obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding sequences and results in very high transgene expression, at least as good as in a control E1 replacement vector.

[0182] To this end, the 2.7 kb *Eco*RI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the *Eco*RI site of pBluescript (KS-) (Stratagene). Next, the *Hind*III site in the polylinker was removed by digestion with *Eco*RV and *Hind*I and subsequent religation. The resulting clone pBS.Eco-Eco/ad5ΔHIII was used to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG GCC AA AGG CGC A-3') and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') were used to amplify a sequence from pBS.Eco-Eco/ad5ΔHIII corresponding to sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3') and 4 (5'-GTC GCT GTA GTT GGA CTG G-3') were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the newly introduced *Nco*I site and subsequently digested with *Xba*I and *Mun*I. This fragment was then ligated into a pBS.EcoEco/ad5ΔHIII vector that had been partially digested with *Xba*I and *Mun*I, generating pBS.EcoEco/ad5ΔHIII.Δgp19K.

[0183] To allow insertion of foreign genes into the *Hind*III and *Bam*HI site, an *Xba*I deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the *Bam*HI sites in the Bluescript polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIII.Δgp19KΔXbaI, contains unique *Hind*III and *Bam*HI sites corresponding to sequences 28733 (*Hind*III) and 29218 (*Bam*HI) in Ad5. After introduction of a foreign gene into these sites, either the deleted *Xba*I fragment is re-introduced, or the insert is re-cloned into pBS.Eco-Eco/ad5ΔHIII.Δgp19K using *Hind*III and, for example *Mun*I. Using this procedure, we have generated plasmids expressing HSV-TK (McKnight (1980) *Nucl. Acid. Res.* 8:5949-5964 and Vincent *et al.* (1996) *Hum. Gene Ther.* 7:197-205), hIL-1α (Esandi *et al.*, (1998) *Gene Therapy* 5:778-788), rat IL-3β (Esandi *et al.*, (1998) *Gene* 211(1):151-158), luciferase (De Wit *et al.*, (1987) *Mol. Cell Biol.* 7:725-737) or LacZ. The unique *Srf*I and *Not*I sites in the pBS.Eco-Eco/ad5ΔHIII.Δgp19K plasmid (with or without an inserted gene of interest) are used to transfer the region containing the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔgp19K (with or without an inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

[0184] Recombinant viruses that are both E1 and E3 deleted are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system which includes: an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of interest, the pWE/Ad-AflII-EcoRI fragment, and the pBr/Ad.Bam-rITRΔgp19K plasmid with or without insertion of a second gene of interest.

[0185] In a non-limiting example we describe the generation and functionality of a recombinant adenovirus containing the murine HSA gene in the E1 region and the firefly luciferase gene in the gp19K region. The luciferase gene was excised from pAd/MLP-Luc (described in EP 0707071) as a *Hind*III-*Bam*HI construct and cloned into the *Hind*III-*Bam*HI sites of pBS.Eco-Eco/ad5ΔHIII.Δgp19KΔXbaI. Then the *Msc*I-*Mun*I fragment containing the luciferase gene was cloned into the corresponding sites of pBS.Eco-Eco/ad5Δgp19K generating pBS.Eco-Eco/ad5Δgp19K.luc. This restores the Eco-Eco fragment, but now with the luciferase gene in the place of gp19K.

[0186] To simplify further manipulation, the internal *Eco*RI sites in the luciferase insert were mutated without making changes to the amino acid sequence of the luciferase gene. One *Eco*RI site flanked the *Hind*III site in the 5' non-coding region of the luciferase insert and the other one was located 588 bp 3' from the starting ATG. A 695 bp PCR product was generated with the following primers: 5'-CGA TAA GCT TAA TTC CTT TGT GTT T-3' and 5'-CTT AGG TAA CCC AGT AGA TCC AGA GGA GTT CAT-3' and digested with *Hind*III and *Bst*EII. This fragment was then ligated to *Hind*III-*Bst*EII digested pBS.Eco-Eco/ad5Δgp19K.luc, replacing the corresponding insert in this vector. The resulting construct is named pBS.EcoEco/ad5Δgp19K.luc². The luciferase gene and part of the E3 region was then excised from this

clone with *SrfI* and *NotI* and introduced in the corresponding sites in pBr/Ad.Bam-rlTR generating clone pBr/Ad.Bam-rlTR Δ gp19K/luc².

[0187] The adapter plasmid pAd5/S1800HSA used for the replacement of E1 in the double insert virus contains the murine HSA gene driven by a retrovirus LTR-based promoter. This adapter plasmid was generated from the pAd5/L420-HSA construct described *infra* by replacement of the promoter sequence. First a PCR product was generated on a retroviral vector based on the MFG-S vector described in WO95/34669 using the same primers as for the amplification of the L420 promoter fragment (described *infra*). This PCR amplifies the sequences corresponding to bp 453-877 in the MFG-S vector. The L420 promoter in pAd5/L420-HSA (Fig. 21) was then exchanged for the PCR fragment using the unique *AvrII* and *HindIII* sites. The resulting construct, pAd5/S430-HSA, was then digested with *NheI* and *ScaI* and the 4504 bp fragment containing the HSA gene, pA sequences, Ad5 sequences and vector sequences to the *ScaI* site in the ampicillin gene was isolated.

[0188] The construct pAd5/S430-HSA also was digested with *XbaI* and *ScaI* and the 1252 bp fragment (containing the remainder of the ampicillin gene, the left ITR and packaging signal from adenovirus and the 5' part of the S430 promoter) was isolated. A third fragment of 1576 bp was isolated from the MFG-S-based retroviral vector following an *XbaI* digestion and contains MFG-S sequences corresponding to bp 695-2271.

[0189] The adapter plasmid pAd5/S1800-HSA was constructed by ligating the three isolated fragments. The double insert virus Ad5/S1800-HSA.E31uc was generated (as described above) by transfection of the following DNA fragments into PER.C6 cells: pAd5/S1800-HSA digested with *EcoRI* and *SalI* (2 μ g) + pWE/Ad.AIII-*EcoRI* (2 μ g) digested with *PacI* and *EcoRI* + pBr/Ad.Bam-rlTR Δ gp19Klac⁴ digested with *Sall*. At occurrence of CPE, the virus was harvested and amplified by serial passages on PER.C6 cells. The activity of this HSA-Luc virus was compared to single insert Δ E1 viruses containing either the S1800-HSA or the CMV-Luc transcription units in the E1 region. A549 cells were seeded at 2×10^5 cells/well and infected 5 hrs later with different amounts of the virus. Two days later transgene expression was measured. Luciferase activity was measured using a luciferase assay system (Promega) and expression of the murine HSA gene was measured with an α -HSA antibody (M1/69, Pharmingen). The results are listed in Table III.

[0190] This experiment shows that using the plasmid-based recombination system, double insert viruses can be made and that both inserts are functional. Furthermore, the luciferase activity of the double insert viruses is comparable to the CMV-driven luciferase activity of the control virus. Therefore, we conclude that the E3 promoter is highly active in A549 cells, even in the absence of E1A proteins.

[0191] In addition to manipulations in the E3 region, changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rlTR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

Example 3

Demonstration of the competence of a synthetic DNA sequence, that is capable of forming a hairpin structure, to serve as a primer for reverse strand synthesis for the generation of double-stranded DNA molecules in cells that contain and express adenovirus genes

[0192] Name convention of the plasmids used:

p plasmid

I ITR (Adenovirus Inverted Terminal Repeat)

C Cytomegalovirus (CMV) Enhancer/Promoter Combination

L Firefly Luciferase Coding Sequence hac, haw Potential hairpin that can be formed after digestion with restriction endonuclease Asp718 in both the correct and in the reverse orientation, respectively (Fig. 15).

[0193] The naming convention is exemplified as follows. pICLhaw is a plasmid that contains the adenovirus ITR followed by the CMV-driven luciferase gene and the Asp718 hairpin in the reverse (non-functional) orientation. Plasmids pICLhac, pICLhaw, pICLI and pICL were generated using standard techniques. The schematic representation of these plasmids is shown in Figs. 16-19.

[0194] Plasmid pICL is derived from the following plasmids:

nt. 1	457 pMLP10 (Levrero <i>et al</i> (1991)) Gene 101:195-202)
nt. 458	1218 pCMV β (Clontech, EMBL Bank No. U02451)
nt. 1219	3016 pMLP.luc (IntroGene, unpublished)
nt. 3017	5620 pBLCAT5 (Stein <i>et al</i> , (1989) <i>Mol. Cell Biol.</i> 9:4531-4).

[0195] The plasmid has been constructed as follows:

[0196] The tet gene of plasmid pMLP10 has been inactivated by deletion of the *Bam*HI-*Sac*I fragment, to generate pBLP10ΔSB. Using primer set PCR/MLP1 and PCR/MLP3 a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic *Sac*I restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes *Eco*RI and *Sgr*AI to generate a 196 bp fragment. Plasmid pMLP10ΔSB was digested with *Eco*RI and *Sgr*AI to remove the ITR. This fragment was replaced by the *Eco*RI-*Sgr*AI-treated PCR fragment to generate pMLP/SAL.

[0197] Plasmid pCMV-Luc was digested with *Pvu*II to completion and recirculated to remove the SV40-derived polyadenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-LucΔAd, the Ad5 ITR was replaced by the *Sac*I-site-flanked ITR from plasmid pMLP/SAL by exchanging the *Xmn*I-*Sac*I fragments. The resulting plasmid, pCMV-LucΔAd/SAL, the Ad5 left terminus and the CMV-driven luciferase gene were isolated as a *Sac*I-*Sma*I fragment and inserted in the *Sac*I and *Hpa*I digested plasmid pBLCATS, to form plasmid pICL. Plasmid pICL is represented in Fig. 19; its sequence is presented in Fig. 20

[0198] Plasmid pICL contains the following features:

nt. 1-457	Ad5 left terminus (Sequence 1-457 of human adenovirus type 5)
nt. 458-969	Human cytomegalovirus enhancer and immediate early promoter (Boshart <i>et al.</i> (1985) <i>Cell</i> 41: 521-530) (from plasmid pCMVβ, Clontech, Palo Alto, USA)
nt. 970-1204	SV40 19S exon and truncated 16/19S intron (from plasmid pCMVβ)
nt. 1218-2987	Firefly luciferase gene (from pMLP.luc)
nt. 3018-3131	SV40 tandem poly-adenylation signals from late transcript, derived from plasmid pBLCAT5)
nt. 3132-5620	pUC12 backbone (derived from plasmid pBLCAT5)
nt. 4337-5191	β-lactamase gene (Amp-resistance gene, reverse orientation)

Plasmids pICLhac and pICLhaw

[0199] Plasmids pICLhac and pICLhaw were derived from plasmid pICL by digestion of pICL with the restriction enzyme *Asp*718. The linearized plasmid was treated with Calf-Intestine Alkaline Phosphatase to remove the 5' phosphate groups. The partially complementary synthetic single-stranded oligonucleotides Hp/asp1 and Hp/asp2 were annealed and phosphorylated on their 5' ends using T4-poly-nucleotide kinase.

[0200] The phosphorylated double-stranded oligomers were mixed with the dephosphorylated pICL fragment and ligated. Clones containing a single copy of the synthetic oligonucleotide inserted into the plasmid were isolated and characterized using restriction enzyme digests. Insertion of the oligonucleotide into the *Asp*718 site will at one junction recreate an *Asp*718 recognition site, whereas at the other junction the recognition site will be disrupted. The orientation and the integrity of the inserted oligonucleotide was verified in selected clones by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the *Asp*718 site close to the 3205 *Eco*RI site) was denoted pICLhac. A clone with the oligonucleotide in the reverse orientation (the *Asp*718 site close to the SV40 derived poly-signal) was designated pICLhaw. Plasmids pICLhac and pICLhaw are represented in Figs. 16 and 17.

[0201] Plasmid pICLI was created from plasmid pICL by insertion of the *Sac*I-*Sgr*AI fragment from pICL, containing the Ad5-ITR into the *Asp*718 site of pICL. The 194 bp *Sac*I-*Sgr*AI fragment was isolated from pICL, and the cohesive ends were converted to blunt ends using *E. coli* DNA polymerase I (Klenow fragment) and dNTP's. The *Asp*718 cohesive ends were converted to blunt ends by treatment with mungbean nuclease. By ligation clones were generated that contain the ITR in the *Asp*718 site of plasmid pICL. A clone that contained the ITR fragment in the correct orientation was designated pICLI (Fig. 18).

[0202] Generation of adenovirus Ad-CMV-hcTK. Recombinant adenovirus was constructed according to the method described in Patent application 95202213. Two components are required to generate a recombinant adenovirus. First, an adapter-plasmid containing the left terminus of the adenovirus genome containing the ITR and the packaging signal, an expression cassette with the gene of interest, and a portion of the adenovirus genome which can be used for homologous recombination. In addition, adenovirus DNA is needed for recombination with the aforementioned adapter plasmid. In the case of Ad-CMV-hcTK, the plasmid PCMV.TK was used as a basis. This plasmid contains nt. 1-455 of the adenovirus type 5 genome, nt. 456-1204 derived from PCMVβ (Clontech, the *Pst*I-*Stu*I fragment that contains the CMV enhancer promoter and the 16S/ 19S intron from simian Virus 40), the Herpes Simplex Virus thymidine kinase gene (described in EP patent application 95202213.5), the SV40-derived polyadenylation signal (nt. 2533-2668 of the SV40 sequence), followed by the *Bgl*II-*Sca*I fragment of Ad5 (nt. 3328-6092 of the Ad5 sequence). These fragments are present in a pMLP10-derived (Levrero *et al.* (1991) *Gene* 101: 195-202) backbone. To generate plasmid pAD-CNWhc-TK, plasmid PCMV.TK was digested with *Cla*I (the unique *Cla*I-site is located just upstream of the TK open reading frame) and dephosphorylated with Calf-Intestine Alkaline Phosphate. To generate a hairpin-structure, the synthetic oligonucleotides HP/*cla*2 and HP/*cla*2 were annealed and phosphorylated on their 5'-OH groups with T4-poly-nucleotide kinase and ATP. The double-stranded oligonucleotide was ligated with the linearized vector fragment and used to transform *E. coli* strain "Sure". Insertion of the oligonucleotide into the *Cla*I site will disrupt the *Cla*I recognition

sites. The oligonucleotide contains a new *Cla*I site near one of its termini. In selected clones, the orientation and the integrity of the inserted oligonucleotide was verified by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the *Cla*I site at the ITR side) was denoted pAd-CMV-hcTK. This plasmid was co-transfected with *Cla*I-digested wild-type adenovirus-type 5 DNA into 911 cells. A recombinant "adenovirus in which the CMV-hcTK expression cassette replaces the E1 sequences was isolated and propagated using standard procedures.

[0203] To study whether the hairpin can be used as a primer for reverse strand synthesis on the displaced strand after replication has started at the ITR, the plasmid pICLhac was introduced into 911 cells, i.e. human embryonic retinoblasts transformed with the adenovirus-E1 region. The plasmid pICLhaw served as a control: it contains the oligonucleotide pair HP/asp 1 and 2 in the reverse orientation but is otherwise completely identical to plasmid pICLhac. Also included in these studies were plasmids pICLI and PICL. In the plasmid pICLI the hairpin is replaced by an adenovirus ITR. Plasmid pICL contains neither a hairpin nor an ITR sequence. These plasmids served as controls to determine the efficiency of replication by virtue of the terminal hairpin structure. To provide the viral products other than the E1 proteins (these are produced by the 911 cells) required for DNA replication the cultures were infected with the virus IG.Ad.MLPI.TK after transfection. Several parameters were being studied to demonstrate proper replication of the transfected DNA molecules. First, DNA extracted from the cell cultures transfected with the aforementioned plasmids and infected with IG.Ad.MLPI.TK virus was analyzed by Southern blotting for the presence of the expected replication intermediates, as well as for the presence of the duplicated genomes. Furthermore, from the transfected and IG.Ad.MLPI.TK infected cell populations, virus was isolated that can transfer a luciferase marker gene into luciferase negative cells and express it.

[0204] Plasmid DNA of plasmids pICLhac, pICLhaw, pICLI and PICL were digested with restriction endonuclease *Sa*I and treated with mungbean nuclease to remove the 4 nucleotide single-stranded extension of the resulting DNA fragment. In this manner a natural adenovirus 5' ITR terminus on the DNA fragment was created. Subsequently, both the pICLhac and pICLhaw plasmids were digested with restriction endonuclease *Asp*718 to generate the terminus capable of forming a hairpin structure. The digested plasmids were introduced into 911-cells, using the standard calcium phosphate co-precipitation technique, four dishes for each plasmid. During the transfection, for each plasmid two of the cultures were infected with the IG.Ad.MLPI.TK virus using 5 infectious IG.Ad.MLPI.TK particles per cell. At twenty-hours post transfection and forty hours post-transfection one Ad.tk-virus-infected and one uninfected culture were used to isolate low molecular-weight DNA using the procedure devised by Hirt (as described in Einerhand *et al*, (1995) *Gene Therapy* 2:336-343). Aliquots of isolated DNA were used for Southern analysis. After digestion of the samples with restriction endonuclease *Eco*RI using the luciferase gene as a probe a hybridizing fragment of approx. 2.6kb were detected in only the samples from the adenovirus-infected cells transfected with plasmid pICLhac. The size of this fragment was consistent with the anticipated duplication of the luciferase marker gene. This supports the conclusion that the inserted hairpin is capable of serving as a primer for reverse strand synthesis. The hybridizing fragment was absent if the IG.Ad.MLPI.TK virus was omitted, or if the hairpin oligonucleotide was inserted in the reverse orientation.

[0205] The restriction endonuclease *Dpn*I recognizes the tetranucleotide sequence 5'-GATC-3', but cleaves only methylated DNA, (that is, only plasmid DNA propagated in, and derived, from *E. coli*, not DNA that has been replicated in mammalian cells). The restriction endonuclease *Mbo*I recognizes the same sequences, but cleaves only unmethylated DNA (namely, DNA propagated in mammalian cells). DNA samples isolated from the transfected cells are incubated with *Mbo*I and *Dpn*I and analyzed with Southern blots. These results demonstrated that only in the cells transfected with the pICLhac and the pICLI plasmids large *Dpn*I-resistant fragments were present, that were absent in the *Mbo*I treated samples. These data demonstrate that only after transfection of plasmids pICLI and pICLhac replication and duplication of the fragments occur.

[0206] These data demonstrate that in adenovirus-infected cells linear DNA fragments that have on one terminus an adenovirus-derived inverted terminal repeat (ITR) and at the other terminus a nucleotide sequence that can anneal to sequences on the same strand, when present in single-stranded form thereby generate a hairpin structure, and will be converted to structures that have inverted terminal repeat sequences on both ends. The resulting DNA molecules will replicate by the same mechanism as the wild-type adenovirus genomes.

Example 4

Demonstration that the DNA molecules that contain a luciferase marker gene, a single copy of the ITR, the encapsidation signal and a synthetic DNA sequence, that is able of forming a hairpin structure, are sufficient to generate DNA molecules that can be encapsidated into virions

[0207] To demonstrate that the DNA molecules, generated in Example 3, containing two copies of the CMV-Luc marker gene can be encapsulated into virions, virus was harvested from the remaining two cultures via three cycles of freeze-thaw crushing and was used to infect murine fibroblasts. Forty-eight hours after infection the infected cells

are assayed for luciferase activity. To exclude the possibility that the luciferase activity has been induced by transfer of free DNA, rather than via virus particles, virus stocks were treated with DNaseI to remove DNA contaminants. Furthermore, as an additional control, aliquots of the virus stocks were incubated for 60 minutes at 56°C. The heat treatment does not affect the contaminating DNA, but does inactivate the viruses. Significant luciferase activity was only found in the cells after infection with the virus stocks derived from IG. Ad. MLPI. TK-infected cells transfected with the pICLhc and PICLI plasmids. Neither in the non-infected cells, nor in the infected cells transfected with the pICLhw and pICL was significant luciferase activity demonstrated. Heat inactivation, but not DNaseI treatment, completely eliminated luciferase expression, demonstrating that adenovirus particles, and not free (contaminating) DNA fragments were responsible for transfer of the luciferase reporter gene.

[0208] These results demonstrate that these small viral genomes can be encapsulated into adenovirus particles and suggest that the ITR and the encapsulation signal are sufficient for encapsulation of linear DNA fragments into adenovirus particles. These adenovirus particles can be used for efficient gene transfer. When introduced into cells that contain and express at least some of the adenovirus genes (namely E1, E2, E4, and L, and VA), recombinant DNA molecules that include at least one ITR, at least part of the encapsulation signal as well as a synthetic DNA sequence, that is capable of forming a hairpin structure, have the intrinsic capacity to autonomously generate recombinant genomes which can be encapsulated into virions. Such genomes and vector system can be used for gene transfer.

Example 5

Demonstration that DNA molecules which contain nucleotides 3510-35953 (namely 9.7-100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion of the same strand of the DNA molecule when present in single-stranded form other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells

[0209] In order to develop a replicating DNA molecule that can provide the adenovirus products required to allow the above-mentioned ICLhc vector genome and alike minimal adenovectors to be encapsulated into adenovirus particles by helper cells, the Ad-CW-hcTK adenoviral vector was developed. Between the CMV enhancer/promoter region and the thymidine kinase gene, the annealed oligonucleotide pair (Table 1) HP/cia 1 and 2 was inserted. The vector Ad-CMV-hcTK was propagated and produced in 911 cell using standard procedures. This vector was grown and propagated exclusively as a source of DNA used for transfection. DNA of the adenovirus AdCMV-hcTK was isolated from virus particles that had been purified using CsCl density-gradient centrifugation by standard techniques. The virus DNA was digested with restriction endonuclease *ClaI*. The digested DNA was size-fractionated on an 0.7% agarose gel and the large fragment was isolated and used for further experiments. Cultures of 911 cells were transfected with the large *ClaI*-fragment of the Ad-CMV-hcTK DNA using standard calcium phosphate co-precipitation techniques. Much like in the previous experiments with plasmid pICLhc, the Ad-CMV-hc replicates starting at the right-hand ITR. Once the I-strand is displaced, a hairpin can be formed at the left-hand terminus of the fragment. This facilitates DNA polymerase elongation of the chain towards the right-hand side. The process proceeds until the displaced strand is completely converted to its double-stranded form. Finally, the right-hand ITR is recreated, and in this location, normal adenovirus replication-initiation and elongation occur. The polymerase reads through the hairpin, thereby duplicating the molecule. The input DNA molecule of 33250 bp, that had on one side an adenovirus ITR sequence and at the other side a DNA sequence that had the capacity to form a hairpin structure is duplicated so that both ends contain an ITR sequence. The resulting DNA molecule consists of a palindromic structure of approximately 66500 bp.

[0210] This structure is detected in low-molecular weight DNA extracted from transfected cells using Southern analysis. The palindromic nature of the DNA fragment can be demonstrated by digestion of the low-molecular weight DNA with suitable restriction endonucleases and Southern blotting with the HSV-TK gene as the probe. This molecule can replicate itself in the transfected cells by virtue of the adenovirus gene products that are present in the cells. In part, the adenovirus genes are expressed from templates that are integrated in the genome of the target cells (namely, the E1 gene products), the other genes reside in the replicating DNA fragment itself. This linear DNA fragment cannot be encapsulated into virions. Not only does it lack all the DNA sequences required for encapsulation, but its size also is much too large to be encapsulated.

Example 6

Demonstration that DNA molecules which contain nucleotides 3503-35953 (viz. 9.7-100 map units of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion the same strand of the DNA

molecule other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells and can provide the helper functions required to encapsidate the pICLI and pICLhac derived DNA fragments

[0211] The purpose of the next series of experiments is to demonstrate that the DNA molecule described in Example 5 can be used to encapsulate the minimal adenovectors described in Examples 3 and 4.

[0212] The large fragment isolated after endonuclease *ClaI*-digestion of Ad-CW-hcTK DNA was introduced into 911 cells (as described in Example 5) together with endonuclease *salI*, mungbean nuclease, endonuclease *Asp718*-treated plasmid pICLhac, or as a control similarly treated plasmid pICLhaw. After 48 hours virus was isolated by freeze-thaw crushing of the transfected cell population. The virus preparation was treated with DNaseI to remove contaminating free DNA. The virus was used subsequently to infect Rat2 fibroblasts. Forty-eight hours post infection the cells were assayed for luciferase activity. Only in the cells infected with virus isolated from the cells transfected with the pICLhac plasmid, and not with the pICLhaw plasmid, was significant luciferase activity demonstrated. Heat inactivation of the virus prior to infection completely abolished the luciferase activity, indicating that the luciferase gene was transfected by a viral particle. Infection of 911 cell with the virus stock did not result in any cytopathological effects, demonstrating that pICLhac was produced without any infectious helper virus being propagated on 911 cells. These results demonstrate that the proposed method can be used to produce stocks of minimal-adenoviral vectors, that are completely devoid of infectious helper viruses that are able to replicate autonomously on adenovirus-transformed human cells or on non-adenovirus transformed human cells.

Example 7

Construction of plasmids for the generation and production of minimal adenoviral vectors

[0213] A minimal adenovirus vector contains as operably linked components the adenovirus-derived cis elements necessary for replication and packaging, with or without foreign nucleic acid molecules to be transferred. Recently, the lower limit for efficient packaging of adenoviral vectors has been determined at 75% of the genome length (Parks and Graham, 1997 [need cite]). To allow flexible incorporation of various lengths of stuffer fragments, a multiple cloning site (MCS) was introduced into a minimal adenoviral vector. To obtain a minimal adenoviral vector according to the invention, the following constructs were made: pAd/L420-HSA (Fig. 21) was digested with *BglII* and *SalI* and the vector-containing fragment was isolated. This fragment contains the left ITR and packaging signal from Ad5 and the murine HSA gene driven by a modified retroviral LTR. The right ITR of adenovirus was amplified by PCR on pBr/Ad BamHI-rITR template DNA using the following primers: **PolyL-ITR**: 5'-AAC-TGC-AGA-TCT-ATCGAT-ACT-AGT-CAA-TTG-CTC-GAG-TCT-AGA-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3' and **ITR-BSN**: 5'-CGG-GAT-CCG-TCG-ACG-CGG-CCG-CAT-CAT-CAA-TAA-TAT-ACC-3'. The amplified fragment was digested with *PstI* and *BamHI* and cloned into pUC119 digested with the same enzymes. After sequence confirmation of correct amplification of the ITR and the MCS, a *BglII-SalI* fragment was isolated and cloned into the *BglII/SalI*-digested pAd/L420-HSA fragment described above. The resulting clone was named pAd/L420-HSA.ITR.

[0214] To be able to manipulate constructs of lengths exceeding 30 kb, the minimal adenoviral vector pAd/L420-HSA.ITR was subcloned in a cosmid vector background. To this end, the cosmid vector pWE15 was modified to remove restriction sites in the backbone pWE15 was digested with *PstI* and fragments of 4 kb and 2.36 kb were isolated from agarose gel and ligated together. The resulting clone, stripped of the SV40 *ori*/early promoter and neomycin resistance coding sequence, was named pWE20. Then, pWE20 was digested with *ClaI* and *HindIII* and the sticky ends were filled in with Klenow enzyme. A 6354 bp blunt fragment was ligated to a phosphorylated *NsiI* linker with the following sequence: 5'-CGATGCATCG-3'. The ligated DNA was phenol/chloroform extracted, precipitated with EtOH to change buffers, and digested with excess *NsiI*. Digested DNA was separated from the linkers by electrophoresis, isolated and religated. The resulting clone was named pWE25. Correct insertion of the *NsiI* linker was confirmed by restriction enzyme digestion and sequencing. To construct the minimal adenoviral vector, pAd/L420-HSA.ITR was digested with *ScaI* and *NofI* and the 2 kb fragment containing part of the ampicillin gene and the adeno ITRs was cloned into pWE25 digested with *ScaI* and *NofI*. The resulting clone was named pMV/L420H (Fig. 24). This clone allows easy manipulation to exchange the promoter and/or gene, and also- allows insertion of DNA fragments of lengths not easily cloned into normal plasmid backbones.

[0215] Plasmid pMV/CMV-LacZ was made by exchanging the L420-HSA fragment (*SnaBI-BamHI*) for a fragment from pcDNA3-nlsLacZ (*NruI-BamHI*) containing the CMV promoter and LacZ coding sequences. pcDNA3-nlsLacZ was constructed by insertion of a *KpnI-BamHI* fragment obtained after PCR amplification of the nlsLacZ coding sequences into pcDNA3 (Invitrogen) digested with *KpnI* and *BamHI*. The PCR reaction was performed on a pMLP.nlsLacZ template DNA using the primers 1: 5'-GGG-GTG-GCC-AGG-GTA-CCT-CTA-GGC-TTT-TGC-AA-3' and 2: 5'-GGG-GGG-ATC-CAT-AAA-CAA-GTT-CAG-AAT-CC-3'. Correct amplification and cloning were confirmed by assaying β -galactosidase expression in a transient transfection experiment on 911 cells.

[0216] The vector pAd/MLPnlslacZ was made as follows: pMLP10 (Levrero *et al.*, (1991) *Gene* 101:195-202) was digested with *Hind*III and *Bam*HI and ligated, in a three-part ligation, to a 3.3 kb *Avr*II-*Bam*HI fragment from L7RH β -gal (Kalderson *et al.*, (1984) *Cell* 499-509), and a synthetic linker with *Hind*III and *Xba*I overhang. The linker was made by annealing two oligonucleotides of sequence 5'-AGC TTG AAT TCC CGG GTA CCT-3' and 5'-CTA GAG GTA CCC GGG AAT TCA-3'. The resulting clone was named pMLP.nlsLacZ/-Ad. Next, pMLP.nlsLacZ/-Ad was digested with *Bam*HI and *Nru*I and the vector containing fragment was ligated to a 2766 bp *Bgl*II-*Sca*I fragment from pAd5SalB (Bernards *et al.*, (1982) *Virology* 120:422-432). This resulted in the adapter plasmid pMLP.nlsLacZ (described in EP 0 707 071).

[0217] Propagation of a minimal adenoviral vector can only be achieved by expression of adenovirus gene products. Expression of adenovirus gene products, at levels high enough to sustain production of large quantities of virus, requires replication of the coding nucleic acid molecule. Usually, therefore, replicating helper viruses are used to complement the minimal adenoviral vectors. The present invention, however, provides packaging systems for minimal adenoviral vectors without the use of helper viruses. One of the methods of the invention makes use of a replicating DNA molecule that contains the 5'-ITR and all adenoviral sequences between bp 3510 and 35938, i.e., the complete adenoviral genome except for the E1 region and the packaging signal. Construct pWE/ Δ Ad.A5' (Fig. 25) is an example of a replicating molecule according to the invention that contains two adenoviral-ITRs. pWE/ Δ Ad.A5'. It has been made in a cosmid vector background from three fragments. First, the 5' ITR from Ad5 was amplified using the following primers:

ITR-EPH: 5'-CGG-AAT-TCT-TAA-TTA-AGT-TAA-CAT-CAT-CAR-TAR-TAT-ACC-3' and

ITR-pIX: 5'-ACG-GCG-CGC-CTT-AAG-CCA-CGC-CCA-CAC-ATT-TCA-GTA-CGT-ACT-AGT-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3'. The resulting PCR fragment was digested with *Eco*RI and *Asd*I and cloned into vector pNEB193 (New England Biolabs) digested with the same enzymes. The resulting construct was named pNEB/ITR-pIX. Sequencing confirmed correct amplification of the Ad5 sequences in the left ITR (Ad5 sequences 1 to 103) linked to the pIX promoter (Ad5 sequences 3511 to 3538) except for a single mismatch with the expected sequence according to GenBank (Accession No. M73260/M29978), i.e., an extra C-residue was found just upstream of the *Afl*II site. This ITR-pIX fragment was isolated with *Eco*RI and *Afl*II and ligated to a *Eco*RI-*Afl*II vector fragment containing Ad5 sequences 3539-21567. The latter fragment was obtained by digestion of pBr/Ad.Cla-Bam (*supra*) with *Eco*RI and partially with *Afl*II. The resulting clone was named pAd/LITR(Δ 5')-BamHI. The final construct pWE/ Δ Ad.A5' was made by ligating cosmid vector pWE15.Pac (*supra*) digested with *Pac*I to pAd/LITR(Δ 5')-BamHI digested with *Pac*I-BamHI and pBr/Ad.Bam-rITR.pac#2 (*supra*) digested with *Pac*I/BamHI (Fig. 25).

[0218] An alternative method to produce packaging systems for minimal adenoviral vectors without the use of helper viruses according to the invention is to use a replicating DNA molecule that contains the complete adenoviral genome except for the E1 region and the packaging signal and in which one of the ITRs is replaced by a fragment containing a DNA sequence complementary to a portion of the same strand other than the ITR and that therefore is able to form a hairpin structure (Fig. 10). In a non-limiting example, said DNA sequence complementary to a portion of the same strand other than the ITR is derived from the adeno-associated virus (AAV) terminal repeat. Such a replicating DNA molecule is made following the same cloning strategy as described for pWE/ Δ Ad.A5', but now starting with the AAV terminal repeat linked to part of the adenoviral pIX promoter. To this end, the adenoviral ITR sequences between the *Hpa*I and *Spe*I sites in construct pNEB/ITR-pIX were exchanged for the AAV ITR by introducing the *Pvu*II/*Xba*I fragment from psub201(+) containing the AAV ITR (Samulski *et al.*, (1989) *J. Virol.* 63:3822-3828). This results in construct pWE/AAV. Δ 5' that replicates in an E1 complementing cell line.

[0219] Another alternative packaging system for minimal adenoviral vectors is described *infra* and makes use of the replication system of SV40. A functional helper molecule according to this method contains at least the adenoviral sequences necessary to sustain packaging of a minimal construct but not the E1 sequences and packaging signal, and preferably also lacking ITRs. This adenovirus-derived entity has to be present on a vector that contains, besides the sequences needed for propagation in bacteria, an origin of replication from SV40 virus. Transfection of such a molecule together with the minimal adenoviral vector, described *supra*, into a packaging cell line (e.g. PER.C6) expressing, besides the E1 proteins, SV40 derived Large T antigen proteins, results in Large T-dependent replication of the adenovirus-derived helper construct. This replication leads to high levels of adenoviral proteins necessary for replication of the minimal adenoviral vector and packaging into virus particles. In this way, there is no sequence overlap that leads to homologous recombination between the minimal adenoviral vector construct and the helper molecule. In addition, there is no sequence overlap that leads to homologous recombination between the helper molecule and minimal adenoviral vector on the one side and the E1 sequence in the packaging cell on the other side.

[0220] Replication of a 40 kb adenoviral construct was investigated in cells expressing SV40 Large T proteins. Hereto, 2×10^6 Cos-1 cells were transfected in a T25 flask with the following constructs complexed with lipofectamine reagent (Life techn.): the 8 kb cosmid vector pWE.pac, the 40.5 kb construct pWE/Ad.AflII-rITR and three clones (#1, #5 and #9) of the 40.6 kb construct pWE/Ad.A5' (described *infra*). Control transfections were carried out with the constructs

pWE.pac and pWE/Ad Affil-rITR digested with *PacI* enzyme and a CMV-LacZ expression vector without the SV40 *ori* sequence. Transfection efficiency was 50% as determined by a separate transfection using the CMV-LacZ vector and X-gal staining after 48 hrs. All cells were harvested 48 hrs. following transfection and DNA was extracted according to the Hirt procedure (as described in Einerhand *et al.*, (1995) *Gene Therapy* 2:336-343). Final pellets were resuspended in 50 μ l TE-RNase (20 μ g/ml) and 10 μ l samples were digested with *MboI* (35 units overnight at 37°C). Undigested samples (5 μ l) and *MboI* digested samples were run on a 0.8% agarose gel, transferred to a nylon filter (Amersham) and hybridized to radioactive probes according to standard procedures. One probe was derived from an 887 bp *DpnI* fragment from the cosmid vector pWE.pac and one was derived from a 1864 bp *BsrGI*-*BamHI* fragment from adenoviral sequences. These probes hybridize to a 337 bp band and a 1416 bp respectively in *MboI* digested material. Input DNA from bacterial origin is methylated and therefore not digested with *MboI*. In this way it is possible to specifically detect DNA that is replicated in eukaryotic cells. Figure 26A shows a schematic presentation of the construct pWE/Ad. Δ 5' and the locations of the SV40 origin of replication, the pWE-derived probe and the adenovirus derived probe. The lower part presents the autoradiograms of the Southern blots hybridized to the adenovirus probe (B) and the pWE probe (C). See legends for explanation of sample loading. These experiments show that all lanes that contain material from Cos-1 cells that are transfected with plasmids harboring an SV40 *ori* contain *MboI* sensitive DNA and show a specific band of the expected length. The bands specific for replication in the lanes with Cos-1 cells transfected with *PacI* digested material (lanes B 17/18 and C 15-18) probably result from incomplete *PacI* digestion. From these experiments it can be concluded that it is possible to replicate large DNA fragments with the SV40 LargeT/*ori* system in eukaryotic cells.

Example 8

[0221] A functional adenovirus helper molecule lacking ITR sequences was constructed starting with the clone pWE/Ad.D5' described *supra*. pWE/Ad.D5' was digested with *BstI*1071 and the 17.5 kb vector-containing fragment was religated to give pWE/Ad.D5'-*BstI*1071. This clone was then used to amplify the 3' part of the adenovirus genome sequences without the right ITR. A 2645 bp PCR fragment was generated using the primers Ad3'/Forw. 5'-CGG AAT TCA TCA GGA TAG GGC GGT GG-3' and Ad3'/Rev. 5'-CGG GAT CCT ATC GAT ATT TAA ATG TTT TAG GGC GGA GTA ACT TG-3'. The amplified fragment was digested with *EcoRI* and *BamHI* and subcloned in pBr322 digested with the same enzymes. After confirmation of correct amplification by sequencing, the 2556 bp *SbfI*-*ClaI* fragment of this clone was recloned in pWE/Ad.D5'-*BstI*1071 digested with the same enzymes. The resulting construct lacks the right ITR and is named pWE/ Δ rl-*BstI*1071. Next, in this clone the left ITR was replaced by a linker with a *PacI* and *AflI* overhang made up by annealing the following primers: PA-plX1 5'-TAA GCC ACT AGT ACG TAC TGA AAT GTG TGG GCG TGG C-3' and PA-plX2 5'-TTA AGC CAC GCC CAC ACA TTT CAG TAC GTA CTA GTG GCT TAAT-3'. This removed the left ITR and restored correct sequence of the pIX promoter. The clone is named pWE/ Δ lTR*BstI*1071. Correct insertion of the double stranded linker was confirmed by sequencing. The deleted *BstI*1071 fragment was then cloned back into pWE/ Δ lTR-*BstI*1071 and the correct orientation was checked by restriction digestion. The resulting clone is named pWE/Ad-H. Following transfection of this DNA molecule into packaging cells that express adenoviral E1 proteins and the SV40 Large T antigen, replication of that molecule takes place resulting in high levels of adenoviral proteins encoded by the adenoviral entity on that molecule.

Example 9

Additional modifications of adapter plasmids

[0222] To enable removal of vector sequences from the left ITR in pAd5/Clip (described in example 2B), this plasmid was partially digested with *EcoRI* and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was annealed to itself resulting in a linker with a *Sall* site and *EcoRI* overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the *EcoRI* site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal. Likewise, the *EcoRI* site in pAd5/Clip has been changed to a *PacI* site by insertion of a linker of the sequence 5'-AATTGTCTTAATTAACCGCAATT-3' (as described in example 2). The pAd5/Clip was partially digested with *EcoRI*, dephosphorylated and ligated to the *PacI* linker with *EcoRI* overhang. The ligation mixture was digested with *PacI* to remove concatamers, isolated from agarose gel and religated. The resulting vector was named pAd5/Clippac. These changes enable more flexibility to liberate the left ITR from the plasmid vector sequences.

The vector pAd5/L420-HSA was also modified to create a *Sall* or *PacI* site upstream of the left ITR. Hereto pAd5/L420-HSA was digested with *EcoRI* and ligated to the above described *PacI* linker. The ligation mixture was digested with *PacI* and religated after isolation of the linear DNA from agarose gel to remove concatamerised linkers. This resulted in adapter plasmid pAd5/L420-HSApac. This construct was used to generate pAd5/L420-HSAsal as follows: pAd5/L420-HSApac was digested with *Scal* and *BsrGI* and the vector fragment was ligated to the 0.3 kb

fragment isolated after digestion of pAd5/Clipsal with the same enzymes

Generation of adapter plasmids AdMire and AdApt

[0223] To create an adapter plasmid that only contains a polylinker sequence and no promoter or polyA sequences. pAd5/L420-HSApac was digested with *AvrII* and *BglIII*. The vector fragment was ligated to a linker oligonucleotide digested with the same restriction enzymes. The linker was made by annealing oligos of the following sequence:

PLL-1: 5' - GCC ATC CCT AGG AAG CTT GGT ACC GGT GAA TTC GCT
AGC GTT AAC GGA TCC TCT AGA CGA GAT CTG G-3' and
PLL-2: 5' - CCA GAT CTC GTC TAG AGG ATC CGT TAA CGC TAG CGA
ATT CAC CGG TAC CAA GCT TCC TAG GGA TGG C-3'.

The annealed linkers were digested with *AvrII* and *BglIII* and separated from small ends by column purification (Qiaquick nucleotide removal kit) according to manufacturers recommendations. The linker was then ligated to the *AvrII/BglIII* digested pAd5/L420-HSApac fragment. A clone, named AdMire, was selected that had the linker incorporated and was sequenced to check the integrity of the insert. Adapter plasmid AdMire enables easy insertion of complete expression cassettes.

An adapter plasmid containing the human CMV promoter that mediates high expression levels in human cells was constructed as follows: pAd5/L420-HSApac was digested with *AvrII* and 5' protruding ends were filled in using Klenow enzyme. A second digestion with *HindIII* resulted in removal of the L420 promoter sequences. The vector fragment was isolated and ligated to a PCR fragment containing the CMV promoter sequence. This PCR fragment was obtained after amplification of CMV sequences from pCMVLacI (Stratagene) with the following primers:

CMVplus: 5' -GATCGGTACCACTGCAGTCGTCGAATATTGGCCATTAGCC-3' and
CMVminA: 5' -GATCAAGCTTCCAATGCACCGTTCCCGGC-3'.

The PCR fragment was first digested with *PstI* (underlined in CMVplus) after which the 3'-protruding ends were removed by treatment with T4 DNA polymerase. Then the DNA was digested with *HindIII* (underlined in CMVminA) and ligated into the above described pAd5/L420-HSApac vector fragment digested with *AvrII* and *HindIII*. The resulting plasmid was named pAd5/CMV-HSApac. This plasmid was then digested with *HindIII* and *BamHI* and the vector fragment was isolated and ligated to the polylinker sequence obtained after digestion of AdMire with *HindIII* and *BglIII*. The resulting plasmid was named AdApt. Adapter plasmid AdApt contains nucleotides -735 to +95 of the human CMV promoter (Boshart et al., 1985; M. Boshart, F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein and W. Schaffner. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41,521-530, 1985). A second version of this adapter plasmid containing a *Sall* site in place of the *PacI* site upstream of the left ITR was made by inserting the 0.7 kb *Scal*-*BsrGI* fragment from pClipsal into AdApt digested with *Scal* and partially digested with *BsrGI*. This clone was named AdApt.sal.

Example 10

Modifications on adenoviral plasmids

Generation of pWE/Ad.AflII-rITRΔE2A:

[0224] Deletion of the E2A coding sequences from pWE/Ad.AflII-rITR (ECACC deposit P97082116) has been accomplished as follows. The adenoviral sequences flanking the E2A coding region at the left and the right site were amplified from the plasmid pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction with the Expand PCR system (Boehringer) according to the manufacturers protocol. The following primers were used:

Right flanking sequences (corresponding Ad5 nucleotides 24033 to 25190):

[0225]

5 Δ E2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG 3'
 Δ E2A.DBP-start: 5'-CCA ATG CAT TCG AAG TAC TTC CTT
 10 CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI (NsiI site is generated in the primer Δ E2A.DBP-start, underlined).

[0226] Left flanking sequences (corresponding Ad5 nucleotides 21557 to 22442):

15 Δ E2A.DBP-stop: 5'-CCA ATG CAT ACG CCG CAG ACG G-3'
 Δ E2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

20 The amplified DNA was digested with BamHI and NsiI (NsiI site is generated in the primer Δ E2A.DBP-stop, underlined). Subsequently, the digested DNA fragments were ligated into SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed the exact replacement of the DBP coding region with a unique NsiI site in plasmid pBr/Ad.Sal-rITR Δ E2A. The unique NsiI site can be used to introduce an expression cassette for a gene to be transduced by the recombinant
 25 vector.

[0227] The deletion of the E2A coding sequences was performed such that the splice acceptor sites of the 100K encoding L4-gene at position 24048 in the top strand was left intact. In addition, the poly adenylation signals of the original E2A-RNA and L3-RNAs at the left hand site of the E2A coding sequences were left intact. This ensures proper expression of the L3-genes and the gene encoding the 100K L4-protein during the adenovirus life cycle.

30 Next, the plasmid pWE/Ad.AfIII-rITR Δ E2A was generated. The plasmid pBr/Ad.Sal-rITR Δ E2A was digested with BamHI and SpeI. The 3.9-Kb fragment in which the E2A coding region was replaced by the unique NsiI site was isolated. The pWE/Ad.AfIII-rITR was digested with BamHI and SpeI. The 35 Kb DNA fragment, from which the BamHI/SpeI fragment containing the E2A coding sequence was removed, was isolated. The fragments were ligated and packaged using λ phage-packaging extracts according to the manufacturer protocol (Stratagene), yielding the plasmid pWE/Ad.AfIII-rITR Δ E2A.
 35

This cosmid clone can be used to generate adenoviral vectors that are deleted for E2A by cotransfection of PacI digested DNA together with digested adapter plasmids onto packaging cells that express functional E2A gene product. Examples of E2A complementing cell lines are described infra.

40 *Generation of pWE/Ad.AfIII-rITRsp*

[0228] The 3' ITR in the vector pWE/Ad.AfIII-rITR does not include the terminal G-nucleotide. Furthermore, the PacI site is located almost 30 bp from the right ITR. Both these characteristics may decrease the efficiency of virus generation due to inefficient initiation of replication at the 3' ITR. Note that during virus generation the left ITR in the adapter plasmid
 45 is intact and enables replication of the virus DNA after homologous recombination.

To improve the efficiency of initiation of replication at the 3' ITR, the pWE/Ad.AfIII-rITR was modified as follows: construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI and then partially digested with AvrII and the 17.9 kb vector containing fragment was isolated and dephosphorylated using SAP enzyme (Boehringer Mannheim). This fragment lacks the adenosequences from nucleotide 35464 to the 3' ITR. Using DNA from pWE/Ad.AfIII-rITR as template
 50 and the primers ITR-EPH:

55 5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3' and
 Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'

a 630 bp PCR fragment was generated corresponding to the 3' Ad5 sequences. This PCR fragment was subsequently cloned in the vector pCR2.1 (Invitrogen) and clones containing the PCR fragment were isolated and sequenced to

check correct amplification of the DNA. The PCR clone was then digested with *PacI* and *AvrII* and the 0.5 kb adeno insert was ligated to the *PacI*/partial *AvrII* digested pBr/Ad.Bam-*rITRpac#2* fragment generating pBr/Ad.Bam-*rITRsp*. Next this construct was used to generate a cosmid clone (as described in example 2) that has an insert corresponding to the adenosequences 3534 to 35938. This clone was named pWE/AfIII-*rITRsp*.

Generation of adenovirus template clones lacking DNA encoding for fiber

[0229] Adenovirus infection is mediated by two capsid proteins fiber and penton. Binding of the virus to the cells is achieved by interaction of the protruding fiber protein with a receptor on the cell surface. Internalisation then takes place after interaction of the penton protein with integrins on the cell surface. At least some adenovirus from subgroup C and B have been shown to use a different receptor for cell binding and therefore have different infection efficiencies on different cell types. Thus it is possible to change the infection spectrum of adenoviruses by changing the fiber in the capsid. The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-*rITR*. First a *NdeI* site was removed from this construct. For this purpose, pBr322 plasmid DNA was digested with *NdeI* after which protruding ends were filled using Klenow enzym. This pBr322 plasmid was then re-ligated, digested with *NdeI* and transformed into *E. coli* DH5 α . The obtained pBr/ Δ *NdeI* plasmid was digested with *ScaI* and *Sall* and the resulting 3193 bp vector fragment was ligated to the 15349 bp *ScaI*-*Sall* fragment derived from pBr/Ad.Bam*rITR*, resulting in plasmid pBr/Ad.Bam-*rITR* Δ *NdeI* which hence contained a unique *NdeI* site. Next a PCR was performed with oligonucleotides

NY-up:

5' - CGA CAT ATG TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-
3'

and

NY-down:

5' - CGA GAC CAC TGC CAT GTT - 3'

During amplification, both a *NdeI* (bold face) and a *NsiI* restriction site (underlined) were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM MgCl₂, and 1 unit of *Elongase* heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of \pm 2200 bp was amplified. This PCR fragment was subsequently purified using GeneClean kit system (Bio101 Inc.). Then, both the construct pBr/Ad.Bam-*rITR* Δ *NdeI* as well as the PCR product were digested with restriction enzymes *NdeI* and *SbfI*. The PCR fragment was subsequently cloned using T4 ligase enzyme into the *NdeI* and *SbfI* digested pBr/Ad.Bam-*rITR* Δ *NdeI*, generating pBr/Ad.Bam*R* Δ Fib.

This plasmid allows insertion of any PCR amplified fiber sequence through the unique *NdeI* and *NsiI* sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous recombination in packaging cells described *infra* using an adapter plasmid, construct pBr/Ad.AfIII-*EcoRI* digested with *PacI* and *EcoRI* and a pBr/Ad.Bam*R* Δ Fib construct in which heterologous fiber sequences have been inserted. To increase the efficiency of virus generation, the construct pBr/Ad.Bam*R* Δ Fib was modified to generate a *PacI* site flanking the right ITR. Hereto, pBr/Ad.Bam*R* Δ Fib was digested with *AvrII* and the 5 kb adeno fragment was isolated and introduced into the vector pBr/Ad.Bam-*rITR.pac#8* (described in example 2) replacing the corresponding *AvrII* fragment. The resulting construct was named pBr/Ad.Bam*R* Δ Fib.pac.

Once a heterologous fiber sequence is introduced in pBr/Ad.Bam*R* Δ Fib.pac, the fiber modified right hand adenovirus clone may be introduced into a large cosmid clone as described for pWE/Ad.AfIII-*rITR* in example 2. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination making the process extremely efficient.

Generation of adenovirus clones lacking DNA encoding hexon

[0230] A major limitation for gene therapy approaches using adenovirus type 5-based recombinant adenoviruses is

the presence of neutralising antibodies in human serum. As much as 80-90% of individuals contain neutralising immunity to Ad5. The majority of the neutralising antibodies is directed to the hexon protein. Hexon proteins from different serotypes show highly variable regions present in loops that are predicted to be exposed at the outside of the virus (Athappilly et al., 1994; J. Mol. Biol. 242, 430-455). Most type specific epitopes have been mapped to these highly variable regions (Toogood et al., 1989; J. Gen. Virol. 70, 3203-3214). Thus replacement of (part of) the hexon sequences with corresponding sequences from a different serotype is an effective strategy to circumvent (pre-existing) neutralising antibodies to Ad5. Hexon coding sequences of adenovirus serotype 5 are located between nucleotides 18641 and 21697.

To facilitate easy exchange of hexon coding sequences from alternative adenovirus serotypes into the adenovirus serotype 5 backbone, first a shuttle vector was generated. This subclone, coded pBr/Ad.Eco-PmeI, was generated by first digesting plasmid pBr322 with EcoRI and EcoRV and inserting the 14 kb PmeI-EcoRI fragment from pWE/Ad.AfIII-Eco. In this shuttle vector a deletion was made of a 1430 bp SanDI fragment by digestion with SanDI and religation to give pBr/Ad.Eco-PmeI ΔSanDI. The removed fragment contains unique SpeI and MunI sites. From pBr/Ad.Eco-PmeI ΔSanDI the adenovirus serotype 5 DNA encoding hexon was deleted. Hereto, the hexon flanking sequences were PCR amplified and linked together thereby generating unique restriction sites replacing the hexon coding region. For these PCR reactions four different oligonucleotides were required: Δhex1-Δhex4.

Δhex1: 5' - CCT GGT GCT GCC AAC AGC - 3'

Δhex2: 5' - CCG GAT CCA CTA GTG GAA AGC GGG CGC GCG - 3'

Δhex3: 5' - CCG GAT CCA ATT GAG AAG CAA CCA ACA TCA ACA AC - 3'

Δhex4: 5' - GAG AAG GGC ATG GAG GCT G - 3'

The amplified DNA product of ± 1100 bp obtained with oligonucleotides Δhex1 and Δhex2 was digested with BamHI and FseI. The amplified DNA product of ± 1600 bp obtained with oligonucleotides Δhex3 and Δhex4 was digested with BamHI and SbfI. These digested PCR fragments were subsequently purified from agarose gel and in a tri-part ligation reaction using T4 ligase enzyme linked to pBr/Ad.Eco-PmeI ΔSanDI digested with FseI and SbfI. The resulting construct was coded pBr/Ad.Eco-PmeI ΔHexon. This construct was sequenced in part to confirm the correct nucleotide sequence and the presence of unique restriction sites MunI and SpeI.

pBr/Ad.Eco-PmeI ΔHexon serves as a shuttle vector to introduce heterologous hexon sequences amplified from virus DNA from different serotypes using primers that introduce the unique restriction sites MunI and SpeI at the 5' and 3' ends of the hexon sequences respectively. The hexon modified sequences are subsequently introduced in the construct pWE/Ad.AfIII-rITR by exchange of the Ascl fragment generating pWE/Ad.AfIII-rITRHexXX where XX stands for the serotype used to amplify hexon sequences.

Generation of adenoviral clones lacking DNA encoding penton

[0231] The adenovirus type 5 penton gene is located between sequences 14156 and 15869. Penton base is the adenovirus capsid protein that mediates internalisation of the virus into the target cell. At least some serotypes (type C and B) have been shown to achieve this by interaction of an RGD sequence in penton with integrins on the cell surface. However, type F adenoviruses do not have an RGD sequence and for most viruses of the A and D group the penton sequence is not known. Therefore, penton may be involved in target cell specificity. Furthermore, as a capsid protein, the penton protein is involved in the immunogenicity of the adenovirus. Individuals, including patients that are candidates for gene therapy approaches, may have pre-existing antibodies directed to penton proteins in their serum. The replacement of Ad5 penton sequences with penton sequences from certain other serotypes therefore, will affect infection specificity as well as immunogenicity of the virus. To be able to introduce heterologous penton sequences in Ad5 we made use of the plasmid-based system described *infra*. First a shuttle vector for penton sequences was made by insertion of the 7.2 kb NheI-EcoRV fragment from construct pWE/Ad.AfIII-EcoRI (described in example 2) into pBr322 digested with the same enzymes. The resulting vector was named pBr/XN. From this plasmid Ad5 penton sequences were deleted and replaced by unique restriction sites that are then used to introduce new penton sequences from other serotypes. Hereto, the left flanking sequences of penton in pBr/XN were PCR amplified using the following primers:

DP5-F: 5' CTG TTC CTC CTC CTA ATA GC - 3' and

DP5-R: 5' - CGC GGA TCC TGT ACA ACT AAG GGG AAT ACA AG - 3'

DP5-R has an BamHI site (underlined) for ligation to the right flanking sequence and also introduces a unique BsrGI site (bold face) at the 5'-end of the former Ad5 penton region. The right flanking sequence was amplified using:

DP3-F: 5'-CGC GGA TCC CTT AAG GCA AGC ATG TCC ATC CTT-3' and
DP3-3R: 5'-AAA ACA CGT TTT ACG CGT CGA CCT TTC-3'

DP3-F has an BamHI site (underlined) for ligation to the left flanking sequence and also introduces a unique AflII site (bold face) at the 3'-end of the former Ad5 penton region. The two resulting PCR fragments were digested with BamHI and ligated together. Then this ligation mixture was digested with AvrII and BglII. pBr/XN was also digested with AvrII and BglII and the vector fragment was ligated to the digested ligated PCR fragments. The resulting clone was named pBr/Ad.Δ penton. Penton coding sequences from serotypes other than Ad5 were PCR amplified such that the 5' and 3' ends contained the BsrGI and AflII sites respectively. Introduction of these heterologous penton sequences in pBr/Ad.Δ penton generates constructs named pBr/Ad.pentonXX where XX represents the number of the serotype corresponding to the serotype used to amplify the inserted penton sequences. Subsequently the new penton sequences were introduced in the pWE/Ad.AflII-rITR construct by exchanging the common FseI fragment. Importantly, in stead of pWE/Ad.AflII-rITR it is also possible to insert the FseI fragment from pBr/Ad.pentonXX into a pWE/Ad.AflII-rITR vector having a modified hexon and/or fiber sequence. In this way the plasmid-based system to generate adenoviruses enables flexible design of any adenovirus with any desired characteristic concerning efficiency and specificity of infection of the target cell as well as immunogenicity.

Example 11

Generation of replicating viruses

[0232] The plasmid-based system to generate recombinant adenoviruses described *infra* is also very well suited to generate replicating viruses. Replicating viruses may be used for gene therapy approaches aimed at eradicating tumor cells. For example suicide gene therapy methods using replicating adenoviruses that express the Herpes simplex virus-1 thymidine kinase gene (HSV-tk) may have improved efficacy due to increased spread of the vector. Safety is ensured by the possibility to block replication at any time by administration of ganciclovir. Replicating viruses expressing HSV-tk or a marker gene have been generated with the double homologous recombination system described in example 2. Hereto, the following constructs are transfected onto packaging cells:

- pBr/Ad.IITR-Sall(9.4), digested with EcoRI and Sall to liberate the adeno insert from the vector sequences.
- pWE/Ad.AflII-EcoRI digested with PacI and EcoRI
- pBr/Ad.Bam-rITRΔp19K/luc² or pBr/Ad.Bam-rITRΔgp19K/TK digested with Sall,

whereby the third construct is a derivative of the pBr/Ad.Bam-rITR construct that is made by replacement of the gp19K coding region with either a marker gene (luciferase) or the HSV-tk gene as described in example 2 for E1-deleted viruses with modifications in the E3 region. In stead of pBr/Ad.Bam-rITR, the modification of the E3 region may also be introduced in pBr/Ad.Bam-rITRpac#2 or #8, or in pBr/Ad.Bam-rITRsp. This enables liberation of the right ITR from the vector sequences by digestion with PacI and increases the efficiency by which viruses are generated.

Example 12

Generation of recombinant viruses using the described plasmid-based system is extremely efficient and reliable

[0233] Several methods have been described previously for the generation of recombinant adenoviruses. One of these methods makes use of a circular large adenoviral plasmid that is cotransfected on packaging cells with a linearised adapter plasmid (Bett *et al.*, 1994). The efficiency of this method is low due to the fact that the ITRs are linked head to head in the large adenoviral plasmid. Other methods make use of a recombination step in specialised bacteria that lead to a recombinant viral DNA clone (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998). After restriction analysis of the clones and selection of correct recombinants a different strain of bacteria has to be transformed to make a large batch of the DNA. Then linearised fragments are transfected in packaging cells and recombinant viruses appear within a week following transfection.

[0234] The plasmid system described *infra* differs from the methods described above. The system combines easy

manipulation of small adapter plasmids in standard bacteria with efficient homologous recombination in packaging cells due to linearised large adenoviral plasmids

The high efficiency of homologous recombination in E1-complementing packaging cells is exemplified by the experiment described below.

A 96-well microtiter tissue culture plate (plate 1) (Greiner, The Netherlands, catalogue #6555180) was first coated with poly-L-lysine (PLL, 0.1 mg/ml) (Sigma) dissolved in sterile water by incubating each well for 20-120 minutes at room temperature. Alternatively, precoated 96-well plates can be used (Becton and Dickinson). After the incubation with PLL, each well was washed two times with 100 µl sterile water and dried at room temperature for at least two hours. The day before transfection PER.C6 cells were harvested using trypsin-EDTA and counted. The cells were then diluted to a suspension of 45,000 cells per 100 µl followed by seeding 100 µl per well of the PLL coated 96-well plates. The next day 2.6 µl of *Sa*/I linearized pAd/CMV-LacZ and 2.6 µl of *Pac*I linearized pWE-Ad.AflIII-rITR plasmid DNA (both 1 µg/µl) and 95 µl serum free Dulbecco's Modified Eagles Medium (DMEM) were mixed with 25.6 µl lipofectamine diluted in 74.4 µl serum free DMEM by adding the lipofectamine to the DNA mix. The DNA/lipofectamine mixture was left at room temperature for 30 minutes after which 1.3 ml serum free media was added. The latter mixture was then added (30 µl per well) to PER.C6 seeded wells that were washed with 200 µl DMEM prior to transfection. After 3 hours in a humidified CO₂ incubator (37°C, 10% CO₂) 200 µl DMEM with 10% fetal calf serum 10 mM MgCl₂ was added to each well and the plates were returned to the humidified CO₂ incubator (37°C, 10% CO₂). The next day the medium of each well was replaced with 200 µl DMEM, 10% FCS, 10 mM MgCl₂. The plates were then left in the humidified CO₂ incubator for an additional three days after which the wells were subjected to freezing at -20°C for at least 1 hour followed by thawing and resuspension by repeated pipetting. Transfection efficiency was determined using lacZ staining in additional plates and found to be approximately 40% for each transfected well of PER.C6 cells. An aliquot of 100 µl of freeze/thawed transfected cells was transferred to each well of a plate with new PER.C6 cells seeded as described above without PLL coated plates (plate 2). The second 96-well plate with PER.C6 cells incubated with freeze/thaw cell lysate of the first transfected plate was checked for CPE. At least 5% of the wells showed clear CPE after 2 days. Four days after infection with the lysate from plate 1 the plate was subjected to one freeze-thaw cycle and 10 µl from each lysed well was added to wells of a plate seeded with A549 cells (1 x 10⁴ cells per well seeded in 100 µl in DMEM, 10% FCS the day before). Two days after infection the wells were stained for lacZ activity. Of the infected wells 96% were infected and stained blue. All wells stained and a large number of wells showed 100% blue staining and thus transduction of all cells with adenoviral vector carrying lacZ. Extrapolated from MOI experiments in tissue culture flasks the adenoviral titer of well-produced virus is around 10⁶-10⁷ infectious units per ml.

From the high percentage of wells that contain LacZ virus we conclude that the plasmid-based system for the generation of adenoviruses described *infra* is very efficient.

In addition to highly efficient, the system is also very reliable. Using the routine procedure of virus generation as described in example 2 (section C) we obtain a T80 flask with adenovirus infected cells showing full CPE. Different stocks of viruses obtained after cotransfection with adapter plasmids carrying different transgenes (Luciferase, LacZ, ratIL-3, humanIL1α, HSV1-TK, ceNOS, hgp100) and different promoters (MLP, CMV, E3 or retroviral LTR) in a total of 16 transfections, were subjected to plaque purification and separate plaques were tested for expression of the transgene. Out of a total of 145 plaques only two were found to be negative. When positive plaques from the first plaque purification were subjected to a second round of plaque purification all tested plaques were found to be positive (144 out of 144 tested). This clearly shows that the plasmid-based system of the invention is very reliable.

Example 13

Generation of minimal adenoviral vectors with large inserts

[0235] In examples 7 and 8 methods are described for the production of minimal adenoviral vectors in E1 expressing packaging cells. The minimal vectors described here only contain an expression cassette for a gene of interest and the adenovirus ITRs and packaging sequences. Efficient packaging of adenoviruses requires a genome length of >27 kb (Parks and Graham, 1997; J. Virol. 71, 3293-3298). Therefore, to be able to produce high titers of minimal adenoviruses it is necessary to include stuffer DNA in the vectors to reach the optimal packaging size. In case one designs a gene correction vector it is possible or even necessary to include a large fragment of genomic DNA homologous to the genome site to be targeted. In other cases the gene of interest may not be large enough to fulfill the packaging size and a stuffer has to be included. Here we describe the construction of larger minimal vectors with stuffer DNA and a method to produce such vectors.

The vectors pMV/L420H (figure 24) and pMV/CMV-LacZ were first modified to create a second NotI site flanking the left ITR. Hereto, pMV/L420H was partially digested with EcoRI and the linear fragment was isolated. This fragment was ligated to a double stranded linker obtained by annealing an oligonucleotide of the sequence 5'-AATTGCGGCCGC-3'. A clone was selected which had the NotI linker inserted in the correct EcoRI site. This clone was named pMV/L420H.

nn. Next pMV/CMV-LacZ was digested with ScaI and BsrGI and the 7 kbp fragment lacking part of the Amp gene and adenovirus ITR was isolated. This fragment was then ligated to the 0.7 kbp ScaI-BsrGI fragment from pMV/L420H.nn. This resulted in pMV/CMV-LacZ.nn. In both minimal vectors pMV/L420H.nn and pMV/CMV-LacZ.nn, the ITRs are flanked by a NotI site. Since the vector backbone is a cosmid vector based on pWE15 these clones can be used to insert large fragments of stuffer DNA. Stuffer inserts can be any piece of DNA that contains no active transcription regions. Alternatively, the described minimal vectors can be used to insert a large fragment of genomic DNA. In case a marker gene is not required, the insertion can be such that the expression cassette for the HSA gene or the LacZ gene is replaced by the genomic fragment by making use of the SnaBI or AvrII site at the 5' end of the expression cassette and the unique sites at the 3' end. One example of a suitable stuffer DNA is a part of the 44th intron of the human Dystrophin genomic DNA (Genbank accession code: M86524). The generation of large cosmid clones containing the above described minimal adenoviral vectors and 31.7 kb of the dystrophin intron sequence are described below. Hereto the dystrophin sequence is digested with XhoI and BstBI and the 31.7 kb fragments is isolated. Part of the fragment is left with sticky ends and part is filled in with Klenow. Then pMV/L420H.nn is digested with XhoI and ClaI and ligated to the dystrophin fragment with sticky ends. pMV/CMV-LacZ.nn is digested with XhoI, blunted with Klenow enzyme and ligated to the blunted dystrophin fragment. Both ligations are packaged as described before. The large clones generated in this way tend to be unstable in bacteria probably due to the large insert and the presence of two inverted terminal repeats of adenovirus. An improved method to generate these large minimal vectors is described below. This method makes use of the powerful system of homologous recombination in packaging cells for the generation of recombinant viruses.

Generation of minimal adenoviral vectors by homologous recombination in packaging cells.

[0236] pMV/CMV-LacZ.nn was digested with XhoI and NsiI and the ends were blunted with T4 DNA polymerase. The linear fragment is isolated and ligated to the approximately 17.5 kbp XhoI/KpnI fragment from the dystrophin intron also blunted with T4 DNA polymerase. Clones were selected that contained the dystrophin fragment in the 5' to 3' direction. This clone, named pMV/CMV-LacZ.Dys5' contains the left ITR and packaging signal of adenovirus in addition to the LacZ expression cassette and the 5' part of the dystrophin insert. A second clone was then made by digestion of pMV/CMV-LacZ.nn with BglII. The linear fragment was then partially digested with NotI and the 6.4 kb vector fragment was blunted with Klenow and isolated. This fragment was then ligated to the 18.8 kb PvuI-BstBI dystrophin fragment blunted with T4 DNA polymerase. Clones were selected that contained the dystrophin fragment in the 5' to 3' direction. This clone, named pMV/Dys3'-ITR contains a dystrophin insert that has 4.5 kb overlap with the dystrophin fragment in pMV/CMV-LacZ.Dys5'.

Minimal adenoviral vectors containing the full length 31.7 kb XhoI-BstBI fragment are generated by cotransfection of adenovirus packaging cells (e.g. PER.C6) with pMV/CMV-LacZ.Dys5' and pMV/Dys3' with an adenoviral helper plasmid as described in this invention, for example pWE/Ad.D5' or pWE/AdH and a SV40 LargeT expression construct (see examples 7 and 8). Hereto, the pMV/CMV-LacZ.Dys5' construct is digested with NotI and BstBI and the pMV/Dys3' is digested with NotI and PvuI to liberate the ITRs from the vector sequences and to minimize the amount of vector DNA attached to the dystrophin insert thereby enabling homologous recombination and replication.

BRIEF DESCRIPTION OF THE DRAWINGS

[0237]

Figure 1. Construction of pBS.PGK.PCRI. pBS.PGK.PCRI encodes the human phosphoglycerate kinase promoter (PGK) operatively linked to adenovirus 5 (Ad5)E1 nucleotides 459-916. To construct this plasmid, Ad5 nucleotides 459-916 were PCR amplified with primers Ea-1 and Ea-2, digested with ClaI and cloned into the ClaI-EcoRI sites of pBluescript (Stratagene), resulting in pBS.PCRI. The PGK promoter was excised from pTN by complete digestion with ScaI and partial digestion with EcoRI and cloned into the corresponding sites of pBS.PCRI, resulting in pBS.PGK.PCRI.

Figure 2. Construction of pIG.E1A.E1B.X. pIG.E1A.E1B.X encodes Ad5 nucleotides 459-5738 (E1A and E1B regions) operatively linked to the human PGK promoter. pIG.E1A.E1B.X also encodes Ad5 pIX protein. pIG.E1A.E1B.X was constructed by replacing the ScaI-BspEI fragment of pAT-X/S with the corresponding fragment of pBS.PGK.PCRI.

Figure 3. Construction of pIG.E1A.NEO. pIG.E1A.NEO encodes Ad5 nucleotides 459-1713 operatively linked to the human PGK promoter. Also encoded is the E1B promoter functionally linked to the neomycin resistance gene (Neo^R) and the hepatitis B virus (HBV) poly(A) signal. In this construct, the AUG codon of the E1B 21 kDa protein functions as the initiation codon of Neo^R. To construct this plasmid, the E1B promoter and initiation codon (ATG) of the E1B 21 kDa protein were PCR amplified with primers Ea-3 and Ep-2, where Ep-2 introduces an NcoI site

(5'-CCATGG) at the 21 kDa protein initiation codon. The PCR product (PCII) was digested with *Hpa*I and *Nco*I and ligated into the corresponding sites of pAT-X/S, producing pAT-X/S-PCR2. The *Nco*I-*Stu*I fragment of pTN containing the Neo^R and a portion of the HBV poly(A) site were ligated into the *Nco*I-*Nru*I sites of pAT-X/S-PCR2, producing pAT-PCR2-NEO. The HBV poly(A) signal was completed by replacing the *Sca*I-*Sal*I fragment of pAT-PCR2-NEO with the corresponding fragment of pTN, producing pAT-PCR2-NEO.p(A), and replacing the *Sca*I-*Xba*I fragment of pAT-PCR2-NEO.p(A) with the corresponding fragment of pIG.E1A.E1B.X, producing pIG.E1A.NEO (Figure 4). Construction of pIG.E1A.E1B, pIG.E1A.E1B contains the Ad5 nucleotides 459-3510 (E1A and E1B proteins) operatively linked to the PGK promoter and HBV poly(A) signal. This plasmid was constructed by PCR amplification of the N-terminal amino acids of the E1B 55 kD protein with primers Eb-1 and Eb-2, which introduces an *Xho*I site, digested with *Bgl*II and cloned into the *Bgl*II-*Nru*I sites of pAT-X/S, producing pAT-PCR3. The *Xba*I-*Xho*I fragment of pAT-PCR3 was replaced with the *Xba*I-*Sal*I fragment (containing the HBV poly(A) site) of pIG.E1A.NEO to produce pIG.E1A.E1B.

Figure 5. Construction of pIG.NEO. pIG.NEO contains the Neo^R operatively linked to the E1B promoter. pIG.NEO was constructed by ligating the *Hpa*I-*Sca*I fragment of pIG.E1A.NEO which contains the E1B promoter and Neo^R into the *Eco*R V-*Sca*I sites of pBS.

Figure 6. Transformation of primary baby rat kidney (BRK) cells by adenovirus packaging constructs. Subconfluent dishes of BRK cells were transfected with 1 or 5 µg of with either pIG.NEO, pIG.E1A.NEO, pIG.E1A.E1B, pIG.E1A.E1B.X, pAd5XhoIC, or pIG.E1A.NEO plus pDC26, which expresses the Ad5 E1B gene under control of the SV40 early promoter. Three weeks post-transfection, foci were visible, cells were fixed, Giemsa stained and the foci counted. The results shown are the average number of foci per 5 replicate dishes.

Figure 7. Western blot analysis of A549 clones transfected with pIG.E1A.NEO and human embryonic retinoblasts (HER cells) transfected with pIG.E1A.E1B (PER clones). Expression of Ad5 E1A and E1B 55 kD and 21 kD proteins in transfected A549 cells and PER cells was determined by Western blot with mouse monoclonal antibodies (Mab) M73 which recognizes E1A gene products and Mabs A1C6 and C1G11, which recognize the E1B 55 kDa and 21 kDa proteins, respectively. Mab binding was visualized using horseradish peroxidase-labeled goat anti-mouse antibody and enhanced chemiluminescence. 293 and 911 cells served as controls.

Figure 8. Southern blot analysis of 293, 911 and PER cell lines. Cellular DNA was extracted, *Hind*III digested, electrophoresed and transferred to Hybond N⁺ membranes (Amersham). Membranes were hybridized to radiolabeled probes generated by random priming of the *Ssp*I-*Hind*III fragment of pAd5.SalB (Ad5 nucleotides 342-2805).

Figure 9. Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates and transfected in duplicate with 5 µg pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours post-transfection, cells were stained with X-Gal and blue cells were counted. Results shown are the mean percentage of blue cells per well.

Figure 10. Construction of adenovirus vector, pMLPI.TK. pMLPI.TK was designed to have no sequence overlap with the packaging construct pIG.E1A.E1B, pMLPI.TK was derived from pMLP.TK by deletion of the region of sequence overlap with pIG.E1A.E1B and deletion of non-coding sequences derived from LacZ. SV40 poly(A) sequences of pMLP.TK were PCR amplified with primers SV40-1, which introduces a *Bam*HI site and SV40-2, which introduces a *Bgl*II site. pMLP.TK Ad5 sequences 2496 to 2779 were PCR amplified with primers Ad5-1, which introduces a *Bgl*II site and Ad5-2. Both PCR products were *Bgl*II digested, ligated, and PCR amplified with primers SV40-1 and Ad5-2. This third PCR product was *Bam*HI and *Afl*III digested and ligated into the corresponding sites of pMLP.TK, producing pMLPI.TK.

Figure 11A-B. New adenovirus packaging constructs do not have sequence overlap with new adenovirus vectors. Regions of sequence overlap between the packaging construct, pAd5XhoIC expressed in 911 cells and adenovirus vector, pMLP.TK, that can result in homologous recombination and the formation of replication competent adenovirus are shown (Panel A). In contrast, there are no regions of sequence overlap between the new packaging construct, pIG.E1A.E1B, expressed in PER.C6 cells, and the new adenovirus vector, pMLPI.TK. (Panel A) or between the new packaging construct, pIG.E1A.NEO and the new adenovirus vector pMLPI.TK (Panel B) that can result in homologous recombination and the formation of replication competent adenovirus.

Figure 12. Generation of recombinant adenovirus, IG.Ad.MLPI.TK. Recombinant adenovirus, IG.Ad.MLPI.TK, was generated by co-transfection of 293 cells, with *Sal*I linearized pMLPI.TK and the right arm of *Cla*I digested, wild-type Ad5 DNA. Homologous recombination between linearized pMLPI.TK and wild-type Ad5 DNA produces IG.Ad.MLPI.TK DNA, which contains an E1 deletion of nucleotides 459-3510. 293 cells transcomplement the deleted Ad5 genome, thereby, permitting replication of the IG.Ad.MLPI.TK DNA and its packaging into virus particles.

Figure 13. Rationale for the design of adenovirus-derived recombinant DNA molecules that duplicate and replicate in cells expressing adenovirus replication proteins. A diagram of the adenovirus double-stranded DNA genome indicating the approximate locations of E1, E2, E3, E4, and L regions is shown. The terminal polypeptide (TP) attached to the 5'-termini is indicated by closed circles. The right arm of the adenovirus genome can be purified by removal of the left arm by restriction enzyme digestion. Following transfection of the right arm into 293 or 911

cells, adenoviral DNA polymerase (white arrow) encoded on the right arm, will produce only single-stranded forms. Neither the double-stranded or single-stranded DNA can replicate because they lack an ITR at one terminus. Providing the single-stranded DNA with a sequence that can form a hairpin structure at the 3'-terminus that can serve as a substrate for DNA polymerase will extend the hairpin structure along the entire length of the molecule. This molecule can also serve as a substrate for a DNA polymerase but the product is a duplicated molecule with ITRs at both termini that can replicate in the presence of adenoviral proteins.

Figure 14. Adenovirus genome replication. The adenovirus genome is shown in the top left. The origins of replication are located within the left and right ITRs at the genome ends. DNA replication occurs in two stages. Replication proceeds from one ITR generating a daughter duplex and a displaced parental single-strand which is coated with adenovirus DNA binding protein (DBP, open circles) and can form a panhandle structure by annealing of the ITR sequences at both termini. The panhandle is a substrate for DNA polymerase (Pol; white arrows) to produce double-stranded genomic DNA. Alternatively, replication proceeds from both ITRs, generating two daughter molecules, thereby obviating the requirement for a panhandle structure.

Figure 15. Potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequence. *Asp718* I digestion of pCLha, containing the cloned oligonucleotides, HP/asp1 and HP/asp2 yields a linear double-stranded DNA with an Ad5 ITR at one terminus and the HP/asp sequence at the other terminus. In cells, expressing the adenovirus E2 region, a single-stranded DNA is produced with an Ad5 ITR at the 5'-terminus and the hairpin conformation at the 3'-terminus. Once formed, the hairpin can serve as a primer for cellular and/or adenovirus DNA polymerase to convert the single stranded DNA to double stranded DNA.

Figure 16. Diagram of pCLhac. pCLhac contains all the elements of pCL (Figure 19) but also contains in the *Asp718* site, the HP/asp sequence in an orientation that will produce the hairpin structure shown in Figure 15, following linearization by *Asp718* digestion and transfection into cells expressing adenovirus E2 proteins.

Figure 17. Diagram of pCLhaw. pCLhaw is identical to pCLhac (Figure 16) with the exception that the inserted HP/asp sequence is in the opposite orientation.

Figure 18. Schematic representation of pCLI. pCLI contains all the elements of pCL (Figure 19) but also contains in the *Asp718* site, an Ad5 ITR.

Figure 19. Diagram of pCL. pCL is derived from the following: (i) nucleotides 1-457, Ad5 nucleotides 1-457 including the left ITR, (ii) nucleotides 458-969, human CMV enhancer and immediate early promoter, (iii) nucleotides 970-1204, SV40 19S exon and truncated 16/19S intron, (iv) nucleotides 1218-2987, firefly luciferase gene, (v) nucleotides 3018-3131, SV40 tandem polyadenylation signals from the late transcript, (vi) nucleotides 3132-5620, pUC12 sequences including an *Asp718* site, and (vii) ampicillin resistance gene in reverse orientation.

Figure 20: Shows a schematic overview of the adenovirus fragments cloned in pBr322 (plasmid) or pWE15 (cosmid) derived vectors. The top line depicts the complete adenovirus genome flanked by its ITRs (filled rectangles) and with some restriction sites indicated. Numbers following restriction sites indicate approximate digestion sites (in kb) in the Ad5 genome.

Figure 21: Drawing of adapter plasmid pAd/L420-HSA

Figure 22: Drawing of adapter plasmid pAd/Clip

Figure 23: Schematic presentation of the generation of recombinant adenoviruses using a plasmid-based system. In the top the genome organization of Ad5 is given with filled boxes representing the different early and late transcription regions, and flanking ITRs. The middle presents the two DNAs used for a single homologous recombination and, after transfection into packaging cells, leading to the recombinant virus (represented at the bottom).

Figure 24: Drawing of minimal adenoviral vector pMY/L420H

Figure 25: Schematic presentation of the cloning steps for the generation of the helper construct pWE/AdA5'

Figure 26: Evidence for SV40-LargeT/ori mediated replication of large adenoviral constructs in Cos-1 cells. A) Schematic presentation of construct pWE/Ad.Δ5' and the location of the SV40 ori sequence and the fragments used to prepare probes. B) Autoradiogram of the Southern blot hybridized to the adenovirus probe. C) Autoradiogram of the Southern blot hybridized to the pWE probe. Lanes 1, marker lane: λ DNA digested with *EcoRI* and *HindIII*. Lane 4 is empty. Lanes 2, 5, 7, 9, 11, 13, 15 and 17 contain undigested DNA and Lanes 3, 6, 8, 10, 12, 14, 16 and 18 contain *MboI* digested DNA. All lanes contain DNA from Cos-1 cells as described in the text transfected with pWE.pac (lanes 2 and 3), pWE/Ad.A5' construct #1 (lanes 5 and 6), #5 (lanes 7 and 8) and #9 (lanes 9 and 10), pWE/Ad.AflII-rITR (lanes 11 and 12), pMV/CMV-LacZ (lanes 13 and 14), pWE.pac digested with *PacI* (lanes 15 and 16) or pWE/Ad.AflII-rITR digested with *PacI* (lanes 17 and 18). Arrows point at the expected positive signal of 1416 bp(B) and 887 bp (C).

[0238] All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

[0239] The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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TABLE I

Primers used for PCR amplification of DNA fragments used for generation of constructs described in this patent application

Ea-1	CGTGTAGTGTATTTATACCCG	PCR amplification Ad5 nt.459 →
Ea-2	TCGTCACTGGTGGAAAGCCA	PCR amplification Ad5 nt.960 ←
Ea-3	TACCCGCCGTCCCTAAATGGC	nt. 1284-1304 of Ad5 genome
Ea-5	TGGACTTGAGCTGTAAACGC	nt. 1514-1533 of Ad5 genome
Ep-2	GCC'TCCATGGAGGTCAGATGT	nt. 1721-1702 of Ad5; introduction of NcoI site
Eb-1	GCTTGAGCCCGAGACATGTC	nt. 3269-3289 of Ad5 genome
Eb-2	CCCCTCGAGCTCAA'TCTGTATCTT	nt. 3508-3496 of Ad5 genome; introduction of XhoI site
SV40-1	GGGGGATCCGAACTTGTTTATTGCAGC	introduction BamHI site (nt.2182-2199 of pMLP.TK) adaptation of recombinant adenoviruses
SV40-2	GGGAGATCTAGACATGATAAGATAC	introduction BglII site (nt.2312-2297 of pMLP.TK)
Ad5-1	GGGAGATCTGTACTGAAATGTGTGGGC	introduction BglII site (nt. 2496-2514 of pMLP.TK)
Ad5-2	GGAGGCTGCAGTCTCCAAACGGCGT	nt.2779-2756 of pMLP.TK
1TR1	GGGGGATCCTCAAATCGTCACCTTCCGT	nt.35737-35757 of Ad5 (introduction of BamHI site)
1TR2	GGGGTCTAGACATCATCAATAATATAC	nt.35935-35919 of Ad5 (introduction of XbaI site)

TABLE I (Cont.)

PCR primer sets to be used to create the Sall and Asp718 sites juxtaposed to the ITR sequences.

PCR/MLP1	GGCGAATTCGTCGACATCAATAATAATACC	(Ad5 nt.10-18)
PCR/MLP2	GGCGAATTCGGTACCATCATCAATAATAATACC	(Ad5 nt.10-18)
PCR/MLP3	CTGTGTACACCGGCGCA	(Ad5 nt.200-184)

Synthetic oligonucleotide pair used to generate a synthetic hairpin, recreates an Asp718 site at one of the termini if inserted in Asp718 site:

HP/asp1	5'-GTACACTGACCTAGTCCCGCCCGGAAAGCCCGGCGGCACTAGGTCAG
HP/asp2	5'-GTACCTGACCTAGTCCCGCCCGGCTTTGCCCGGCGGCACTAGGTCAGT

Synthetic oligonucleotide pair used to generate a synthetic hairpin, contains the ClaI recognition site to be used for hairpin formation.

HP/cIa1	5'-GTACATTGACCTAGTCCCGCCCGGCAAGCCCGGCGGCACTAGGTCATCGAT
HP/cIa2	5'-GTACATCGATTGACCTAGTCCCGCCCGGCTTTGCCCGGCGGCACTAGGTCAT

TABLE II
Production of recombinant adenoviral vector
or different packages, cell lines

Cell	Passage Number	IGAd.CMV.lacZ	Yields x 10 ⁻⁶ p.f.u./T175 flask. ¹			dl313	Producer Mean
			IGAd.CMV.TK	IG.Ad.MLPI.TK			
293		6.0	5.9	24		34	17.5
911		8	14	34		180	59.5
PER.C3	17	8	11	44		40	25.8
PER.C5	15	6	17	36		200	64.7
PER.C6	36	10	22	58		320	102

NOTE:

The yields are the mean of two different experiments. IG.Ad.CMV.lacZ and IG.Ad.CMV.TK are described in patent application EP 95 20 2213. The construction of IG.Ad.MLPI.TK is described in this patent application. Yields of virus per T80 flask were determined by plaque assay on 911 cells, as described (Fallaux et al (1996) Hum. Gene Ther. 7: 215-222). #1493).

TABLE III

Double insert viruses with different transgenes replacing the
E1 and E3/gp19K regions express both transgenes in human A549 cells

Virus ¹	Amount	% of cells with HSA expression	Luciferase activity (light units)
IGAd/CMV Luc	5 x 10 ⁷ i.u.	ND	25,726,074
	2.5 x 10 ⁷ i.u.	ND	7,996,542
IGAd/S1800-HSA	100 µl ccl	88%	ND
	50 µl ccl	82%	ND
IGAd/S1800-HAS.E3luc	1.2 x 10 ⁷ i.u.	97%	32,451,300
	6 x 10 ⁷ i.u.	97%	24,716,586
	1.2 x 10 ⁸ i.u.	100%	13,294,321

NOTE:

All virus preps were clarified crude cell lysates (ccl).
A clarified crude cell lysate was made by harvesting cells
with medium at full CPE followed by three freeze/thaw cycles.
pAd/S1800-HSA was not titrated.

Claims

1. A method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of combining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof.
2. A method for generating an adenovirus vector comprising welding together through homologous recombination two nucleic acid molecules comprising partially overlapping sequences wherein said overlapping sequences allow essentially only one homologous recombination which leads to the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof.
3. A method according to claim 1 or claim 2, wherein both of said nucleic acid molecules comprise only one adenovirus inverted terminal repeat or a functional part, derivative and/or analogue thereof.

4. A method according to anyone of claims 1-3, wherein said welding together is performed in a cell or a functional part, derivative and/or analogue thereof.
5. A method according to claim 4, wherein said cell is a mammalian cell.
6. A method according to claim 5, wherein said nucleic acid molecules are not capable of replicating in said mammalian cell prior to said welding together.
7. A method according to anyone of claims 1-6, wherein one of said nucleic acid molecules is relatively small and the other is relatively large.
8. A method according to anyone of claims 1-7, wherein at least one of said nucleic acid molecules provided to said cell comprises an adenovirus inverted terminal repeat which on one side is essentially free of other nucleic acid.
9. A method according to claim 8, wherein said adenovirus inverted terminal repeat is made essentially free of other nucleic acid on one side using a restriction enzyme.
10. A method according to claim 9, wherein said restriction enzyme acts on a site which is not present in adenovirus vector nucleic acid in said nucleic acid molecule.
11. A method according to anyone of claims 4-10, wherein the nucleic acids present in said cell do not comprise sequence overlap that can lead to the formation of replication competent adenovirus.
12. A method according to anyone of claims 4-11, wherein the chromosomal nucleic acid in said cell comprises at least a functional part of an adenovirus E1-region, or a functional derivative and/or analogue thereof.
13. A method according to anyone of claims 4-12, wherein said cell is a PER.C6 cell (ECACC deposit number 96022940) or a functional derivative and/or analogue thereof.
14. A method according to anyone of claims 4-13, wherein said nucleic acid in said cell further comprises a nucleic acid encoding an adenovirus E2-region and/or an adenovirus E4-region protein.
15. A method according to anyone of claims 1-14, wherein at least one of said nucleic acid molecules is linear.
16. A method according to anyone of claims 1-15, wherein at least one of said molecules comprises adenovirus capsid protein encoding nucleic acid derived from two different adenovirus serotypes.
17. A method according to anyone of claims 1-16, wherein said welding together of said nucleic acid molecules leads to the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal, a nucleic acid encoding at least one adenovirus E1-region protein, at least one adenovirus E2-region encoded protein and/or at least one adenovirus E4-region encoded protein and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof and wherein at least one of said E1-region encoded proteins is under transcriptional control of a conditionally active promoter.
18. A recombinant nucleic acid deposited at the ECACC under No. P97082122, No. P97082119, No. P97082117, No. P97082114, No. P97082120, No. P97082121, No. P97082116, No. P97082115 or No. P97082118 or a functional part, derivative and/or analogue thereof.
19. A recombinant nucleic acid pWE/Ad.AflIII-EcoRI, pAd5/CLIP, pAd5/L420-HAS, pBS.Eco-Eco/ad5ΔHIIIΔgpl9KΔXbaI, pMV/L420-H, pMV/CMV-LacZ, pWE/Ad.A5', pWE/AAV.A5', pWE/Ad-H.
20. A recombinant nucleic acid comprising:
adenovirus derived nucleotides 1-454 and adenovirus nucleotides 3511-6095 shown in Figures 21 and 22.
21. A recombinant nucleic acid comprising:
a deletion in an E3 region of a recombinant nucleic deposited at the ECACC under No. P97082122, No. P97082119, No. P97082120, No. P97082116.

22. A recombinant nucleic acid according to claim 21, wherein said deletion comprises a gp19K region.

23. A recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises sufficient adenovirus sequences necessary for replication and capsid gene expression, wherein said nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus.

24. A recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises sufficient adenovirus sequences necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid polymerase, wherein said nucleotide sequence comprises a deletion of one Inverted Terminal Repeat, the E1 region and the encapsulating signal of said adenovirus.

25. A recombinant nucleic acid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenoviral sequences necessary for replication, wherein said nucleotide sequence comprises at least a deletion of the E1 region and encapsulating signal of said adenovirus.

26. A recombinant nucleic acid according to claim 25, wherein said nucleotide sequence further comprises a deletion of at least one of the Inverted Terminal Repeats of said adenovirus.

27. A recombinant nucleic acid according to claim 25 or claim 26, wherein said sequence for adenovirus-independent replication comprises an SV40 origin of replication.

28. A recombinant nucleic acid according to anyone of claims 18-27 wherein said nucleotide sequence comprises no sequences which allow for homologous recombination leading to replication competent virus in a cell into which said recombinant nucleic acid is transferred.

29. An adapter plasmid comprising:

a nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises in operable configuration at least one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination and the generation of a replication-defective, recombinant adenovirus genome.

30. An adapter plasmid according to claim 29, comprising no sequences which allow for homologous recombination leading to replication competent virus in a cell into which said adapter plasmid is transferred.

31. An adapter plasmid according to claim 30, having no E1 region sequences.

32. An adapter plasmid according to anyone of claims 29-31, further comprising a nucleic acid of interest such as a multiple cloning site and/or a transgene.

33. A recombinant nucleic acid according to anyone of claim 18-28 or an adapter plasmid according to anyone of claims 29-32, wherein said transgene is operatively linked to an E3 promoter.

34. A method for the generation of recombinant adenovirus having an E1 deletion and a gp19K deletion, comprising the steps of:

growing a cell comprising adenovirus complementing sequences transfected with

i) an adapter plasmid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises in operable configuration one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination leading to the generation of a replication-defective, recombinant adenovirus genome in a cell into which said adapter plasmid is transferred and having no E1 region sequences, and

ii) a recombinant nucleic acid comprising at least one second nucleotide sequence based on or derived from

an adenovirus, wherein said at least one second nucleotide sequence comprises one Inverted Terminal Repeat and sufficient adenovirus sequences for replication and a partial overlap with said adapter plasmid, wherein said at least one second nucleotide sequence comprises a deletion of at least the E1 region, encapsulating signal and gp19K sequences; wherein said complementing sequences, said first nucleotide sequence and said at least one second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus under conditions whereby recombinant adenovirus having an E1 deletion and a gp19K deletion is generated.

35. A method according to claim 34, wherein said adapter plasmid further comprises a first heterologous nucleotide sequence inserted into said E1 region deletion and said recombinant nucleic acid further comprises a second heterologous nucleotide sequence inserted into said gp19K region.

36. A method for the generation of recombinant adenovirus, comprising the steps of:

growing a cell comprising adenovirus complementing sequences transfected with

- i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and
- ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises sufficient adenovirus sequences for replication, wherein said second nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated.

37. A method for the generation of recombinant adenovirus, comprising the steps of:

growing a cell comprising adenovirus complementing sequences transfected with

- i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and
- ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenoviral sequences necessary for replication, wherein said second nucleotide sequence comprises at least a deletion of the E1 region and encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated.

38. A method according to claim 37, wherein said cell comprises at least one nucleic acid molecule whereby said cell expresses SV40 Large T antigen proteins or functional fragments thereof.

39. A method according to claim 37 or claim 38, wherein said second recombinant nucleic acid molecule is replicated.

40. A replication defective adenovirus comprising:

a genome based on or derived from an adenovirus, wherein said genome comprises at least a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof and wherein said genome comprises no functional adenovirus genes and has no overlapping sequences which

allow for homologous recombination leading to replication competent virus in a cell into which said replication defective adenovirus is transferred.

41. A replication defective adenovirus according to claim 40, further comprising:
one or more nucleic acids of interest

42. A non-human cell comprising a genome of a replication defective adenovirus according to claim 40 or claim 41

43. A non-human cell according to claim 42, wherein said cell is a mammalian cell.

44. A method for transducing a cell, comprising the step of:
contacting said cell with a replication defective adenovirus according to claim 40 or claim 41 under conditions whereby said cell is transduced

45. A non-human cell produced according to the method of claim 44, wherein said cell is a mammalian cell

46. A method for generating recombinant adenovirus comprising the step of:
growing a cell comprising adenovirus complementing sequences and

i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and,

ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said nucleotide sequence comprises at least all adenovirus sequences, or functional fragments or derivatives thereof necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid polymerase, wherein said second nucleotide sequence comprises a deletion of one Inverted Terminal Repeat, the E1 region and the encapsulating signal of said adenovirus;

wherein, said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus,
under conditions whereby recombinant adenovirus is generated.

47. A cell comprising a recombinant nucleic acid according to any one of claims 18-28, 33 or a replication defective adenovirus vector according to anyone of claims 40, 41, 55 or 56 and/or an adapter plasmid according to anyone of claims 29-32.

48. A method for the replacement of a defective gene in a host cell genome comprising the step of:
growing said host cell with a recombinant nucleic acid molecule derived from a replication defective adenovirus comprising a functional version or part thereof of said defective gene under conditions whereby at least one allele of said defective gene in said host cell genome is replaced.

49. A method according to claim 44, wherein said replication defective adenovirus expresses no adenoviral genes.

50. A method according to claim 44, wherein said defective gene is a defective tumor suppressor gene.

51. An isolated cell comprising a genome of a replication-defective adenovirus according to any one of claims 40, 41, 55 or 56.

52. A isolated cell according to claim 51, wherein said cell is a human cell.

53. A recombinant nucleic acid according to anyone of claims 18-28, wherein said deletion in the E3 region is replaced with a transgene.

54. A method according to claim 34 or claim 35, wherein said at least one second nucleotide sequence comprises a

first and second molecule wherein said first molecule has said partial overlap with said adapter plasmid at the 3' end, and said second molecule comprises said Inverted Terminal Repeat and region including deletion of said gp19K sequences.

- 5 55. A replication-defective adenovirus comprising: a genome based on or derived from an adenovirus, wherein said genome comprises a first deletion, in and E1 region and a second deletion in a gp19K region.
56. A replication-defective adenovirus according to claim 55, wherein transcription of said transgene is directed by an E3 promoter.
- 10 57. An isolated cell comprising:
a recombinant nucleic acid according to any one of claims 18-26, 33 or a replication defective adenovirus vector according to anyone of claims 40, 41, 55 or 56 and/or an adapter plasmid according to anyone of claims 29-32.
- 15 58. An isolated cell according to claim 57, wherein said cell is a human cell.

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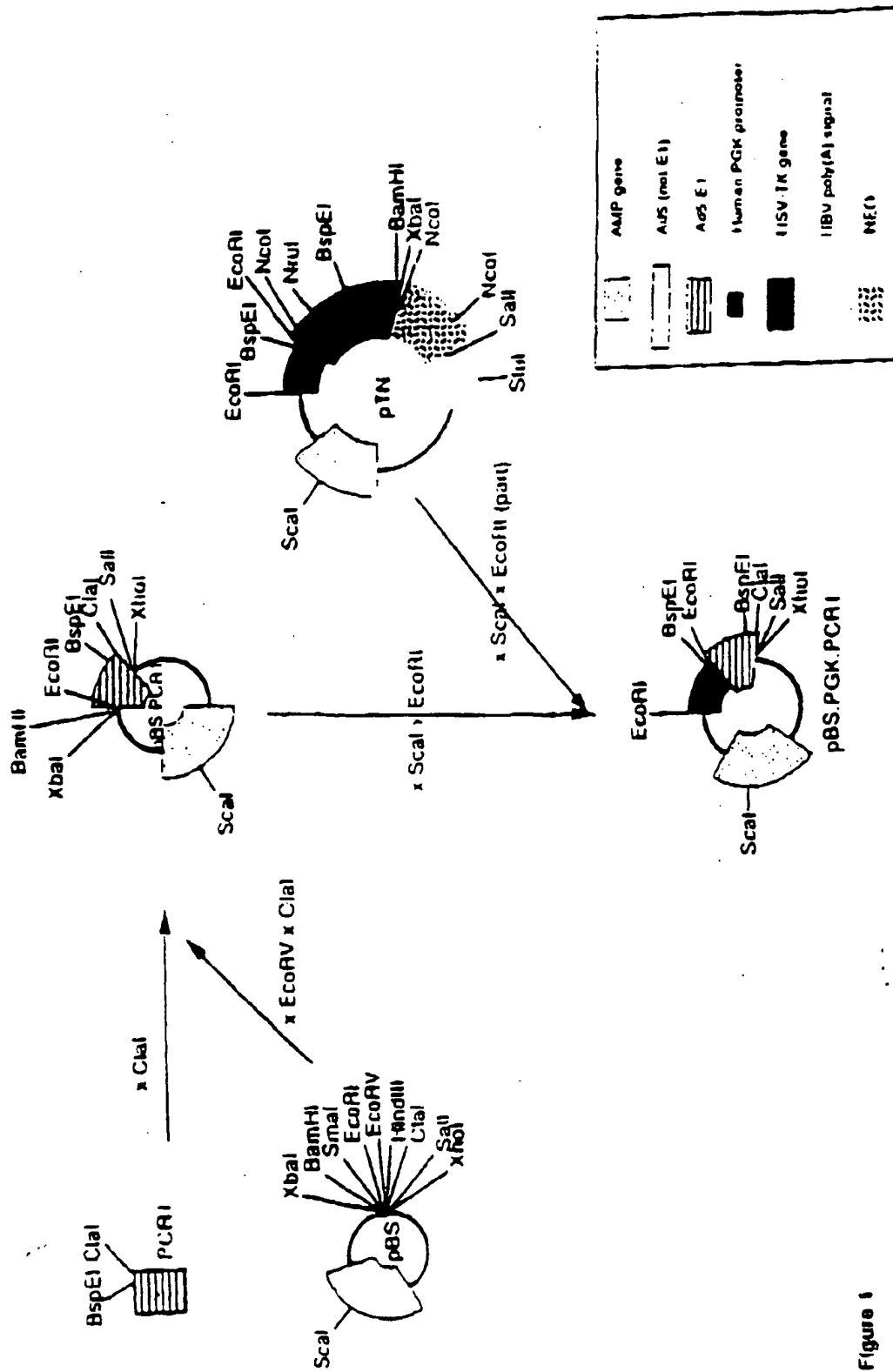


Figure 1
Construction of pBS.PGK.PCRA1

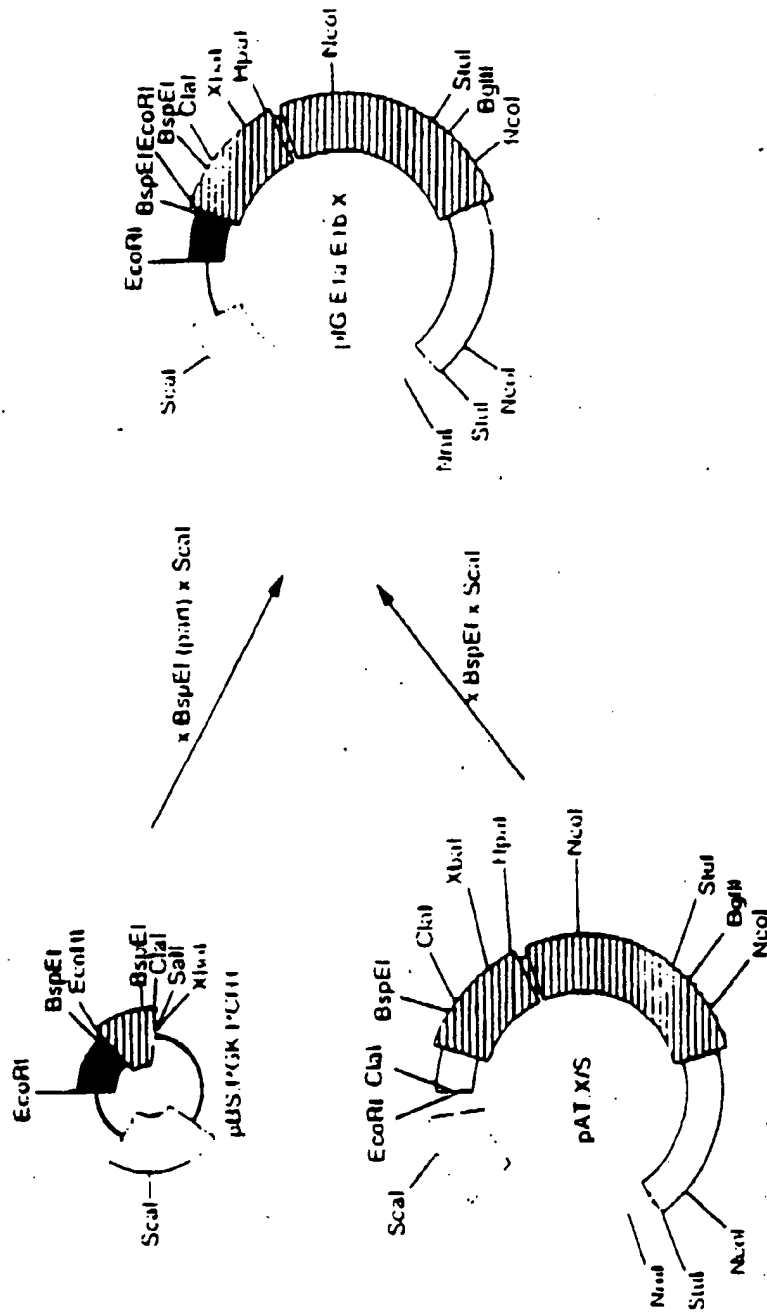


Figure 2

Construction of plG.E1a.E1b.X

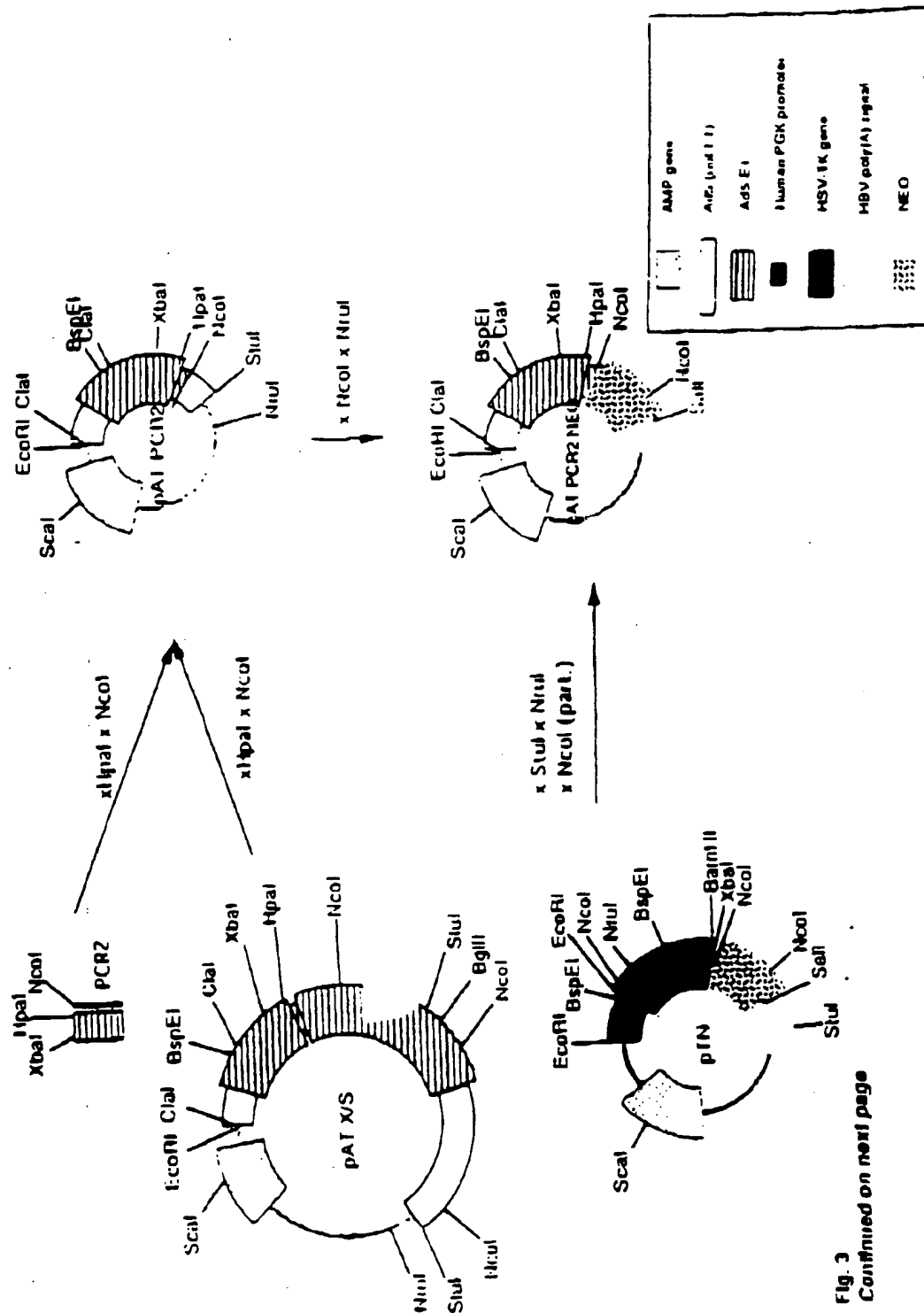


Fig. 3
Continued on next page

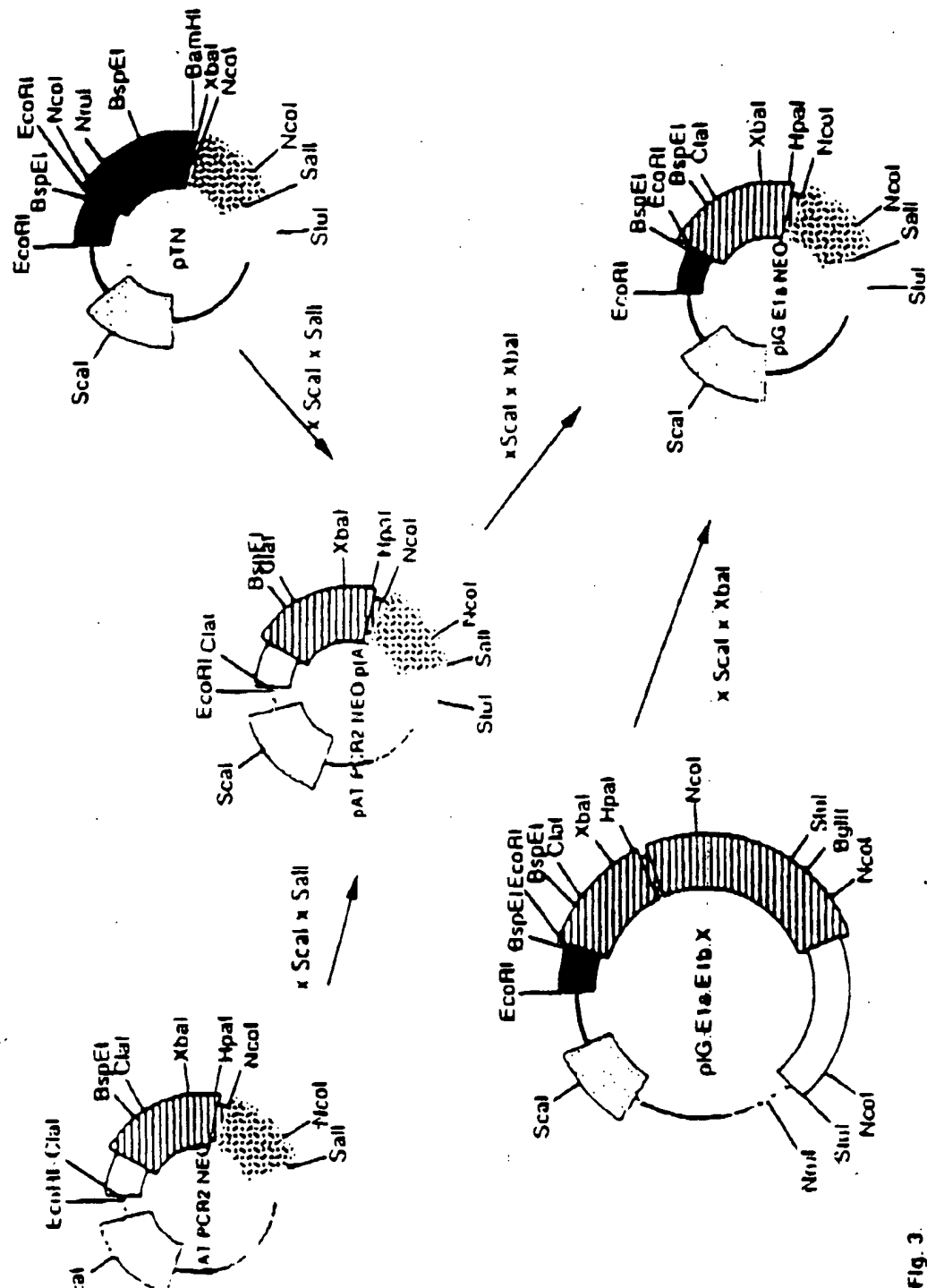


Fig. 3
Construction of pIG.E1a.NEO

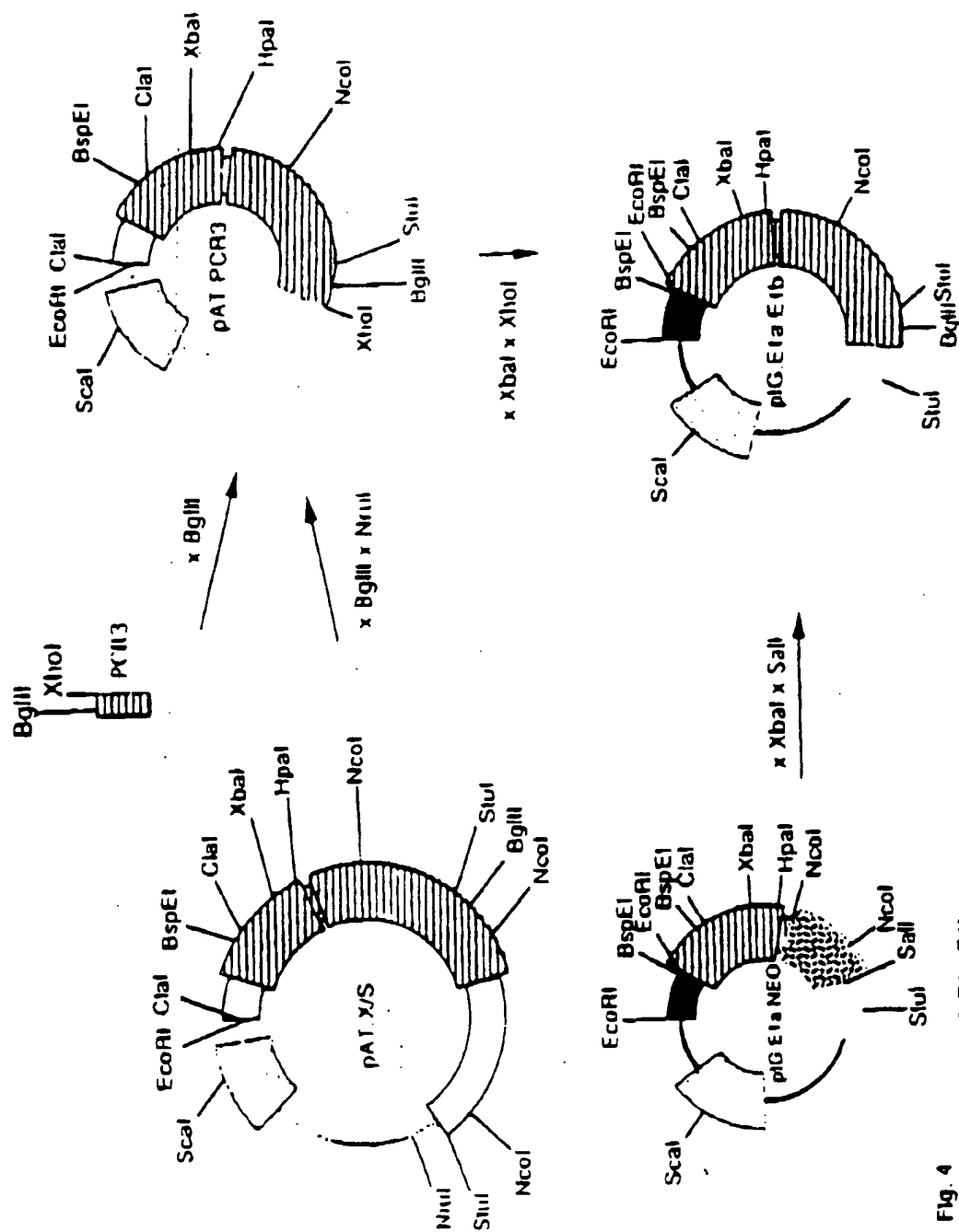


Fig. 4
Construction of pG.E1a.E1b

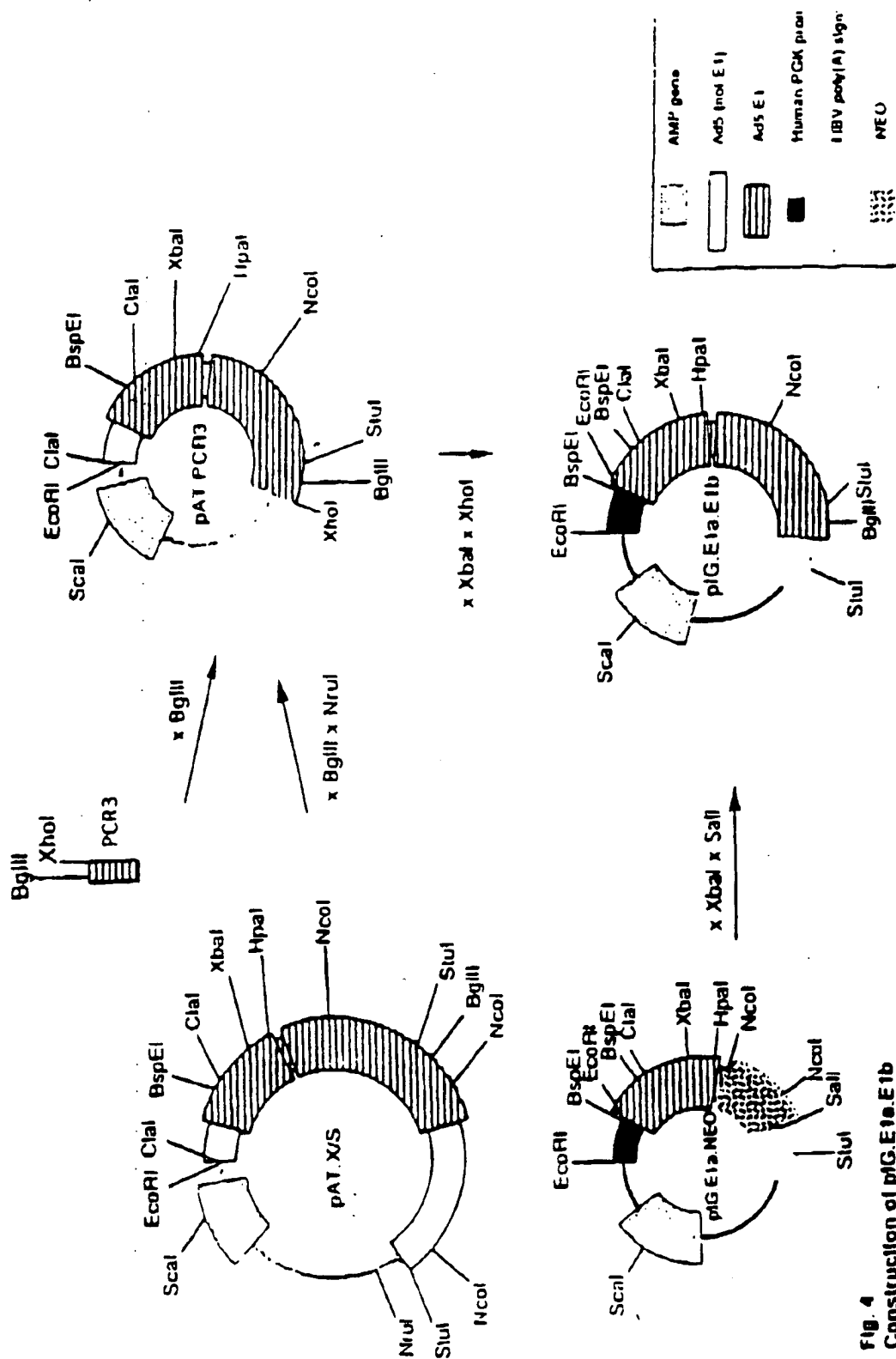


Fig. 4
Construction of pG.E10.E1b

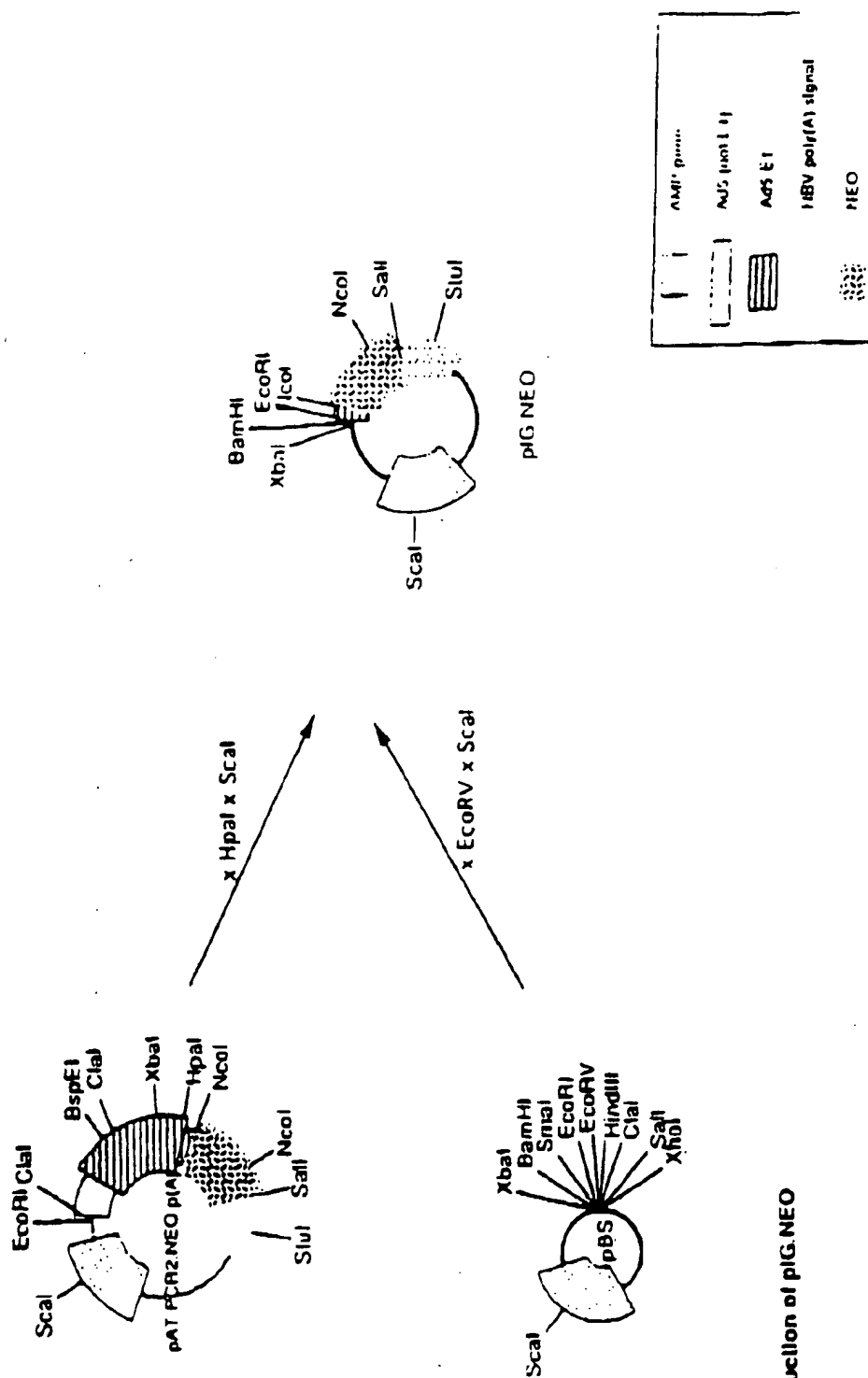


Fig. 5
Construction of pIG.NEO

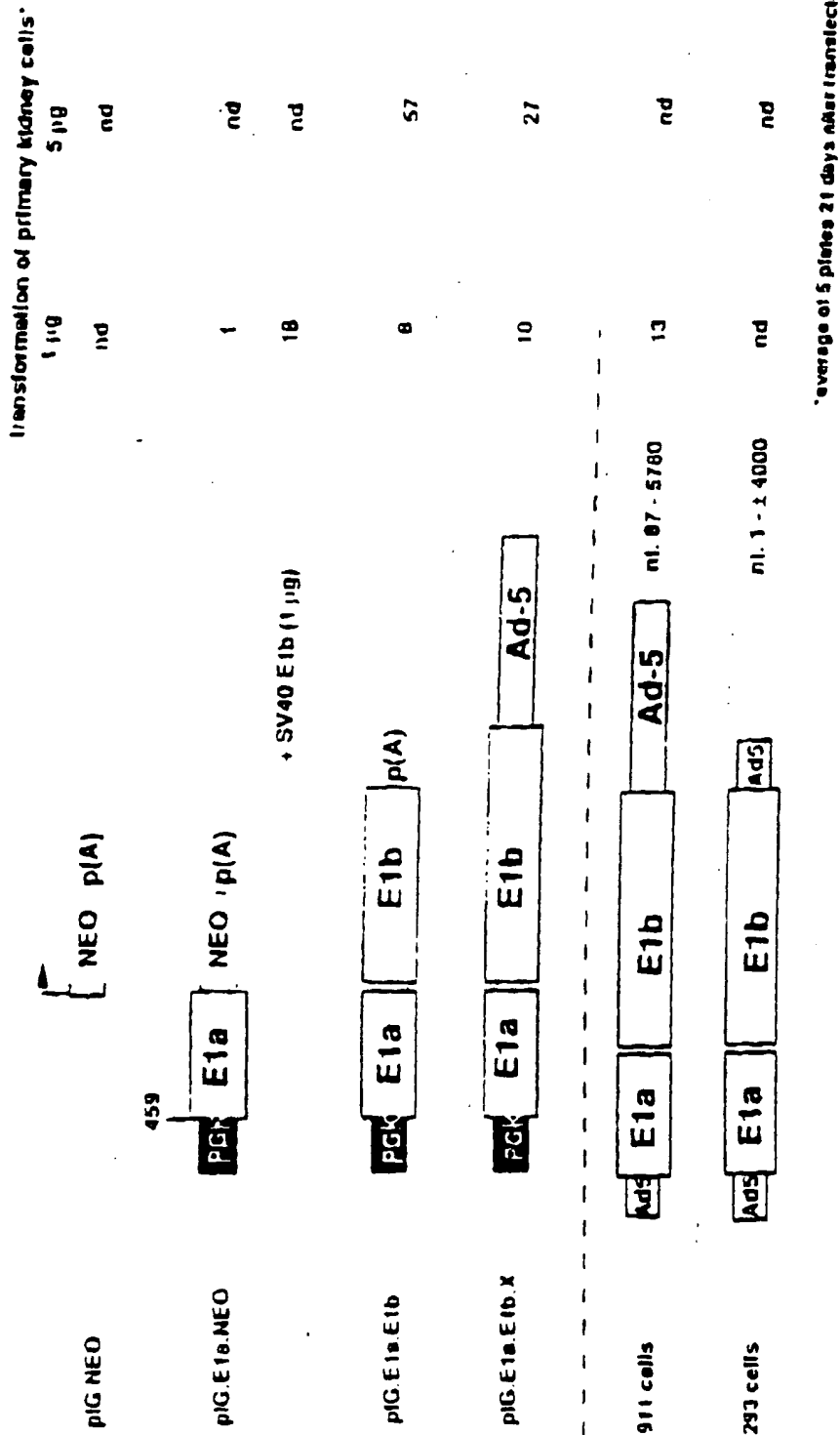


Figure 6
Overview of available adenovirus packaging constructs and assessment of their capacity to transform primary kidney cells

Figure 7

Western blotting analysis of A549 clones transfected with pIG.E1A.NEO and PER clones (HER cells transfected with pIG.E1A.E1B)

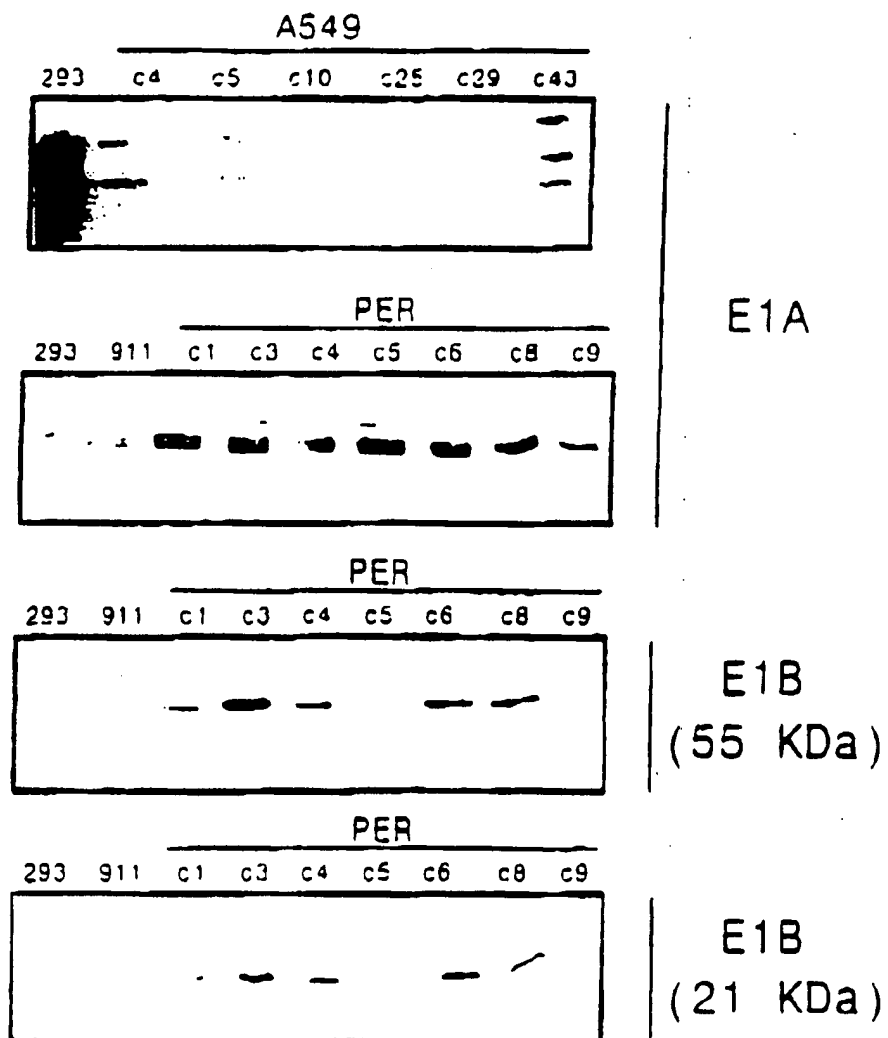


Figure 8

Southern blot analyses of 293, 911 and PER cell lines

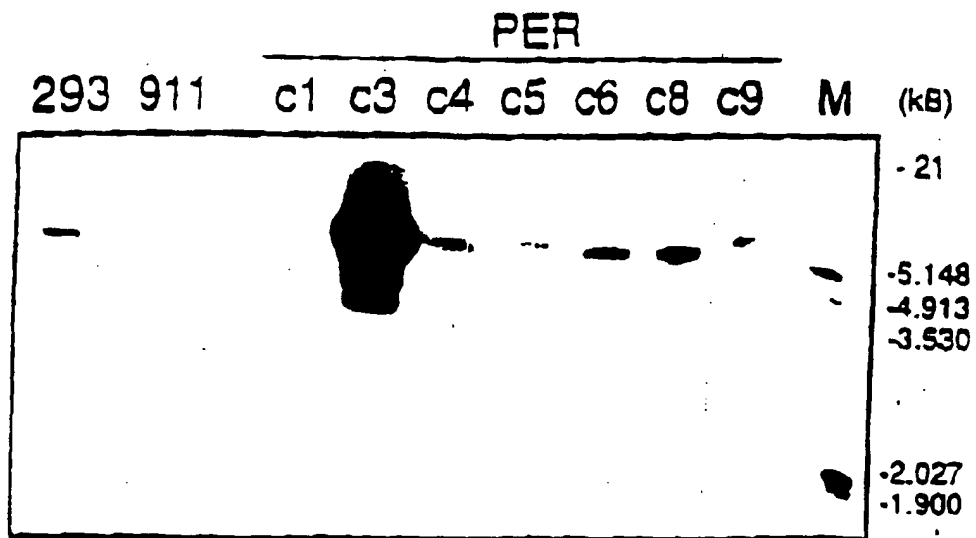
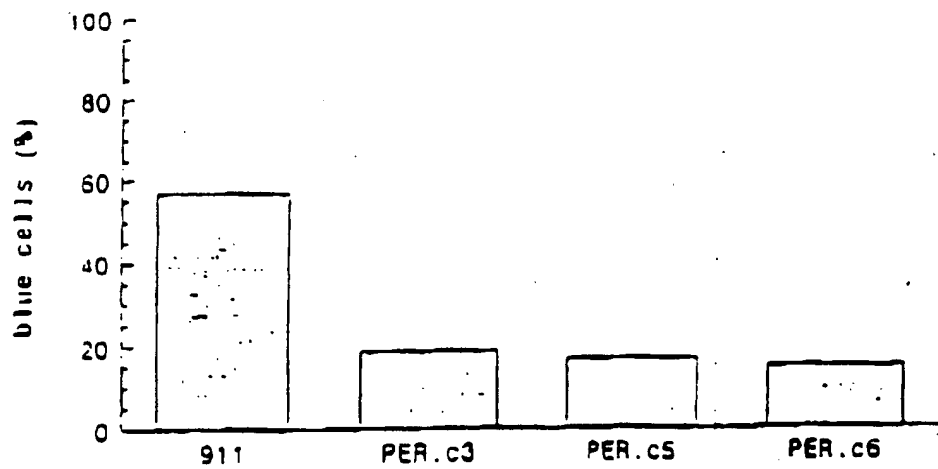


Figure 9

Transfection efficiency of PER.C3, PER.C5, PER.C6 and 911 cells. Cells were cultured in 6-well plates and transfected (n=2) with 5 μ g pRSV.lacZ by calcium-phosphate co-precipitation. Forty-eight hours later the cells were stained with X-GAL. The mean percentage of blue cells is shown.



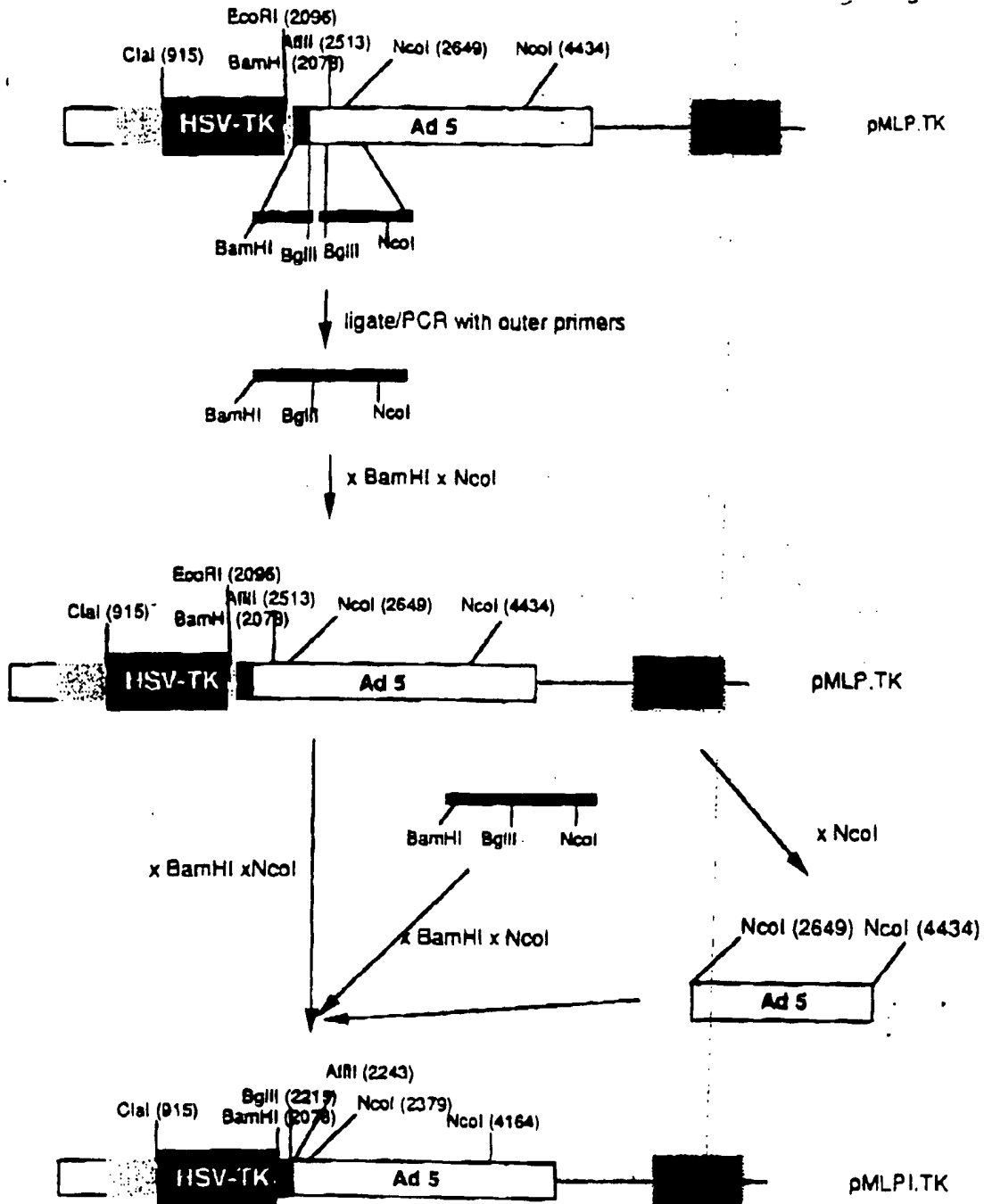


Fig. 10

new recombinant adenoviruses and packaging constructs without sequence overlap

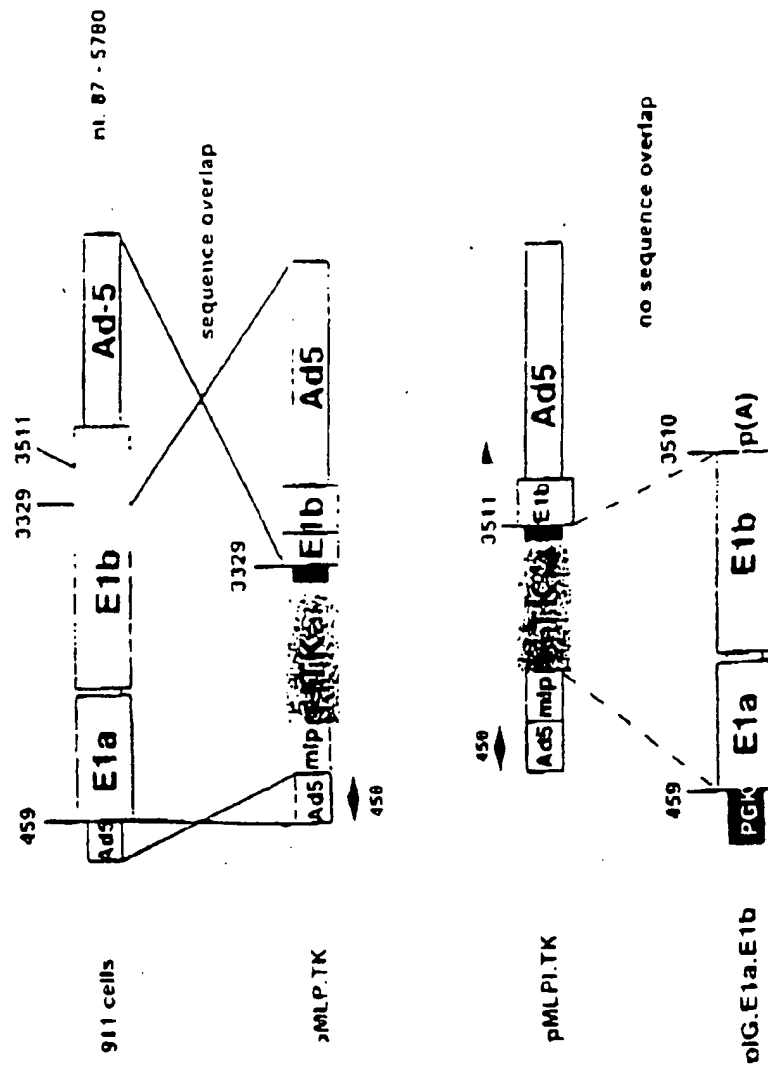


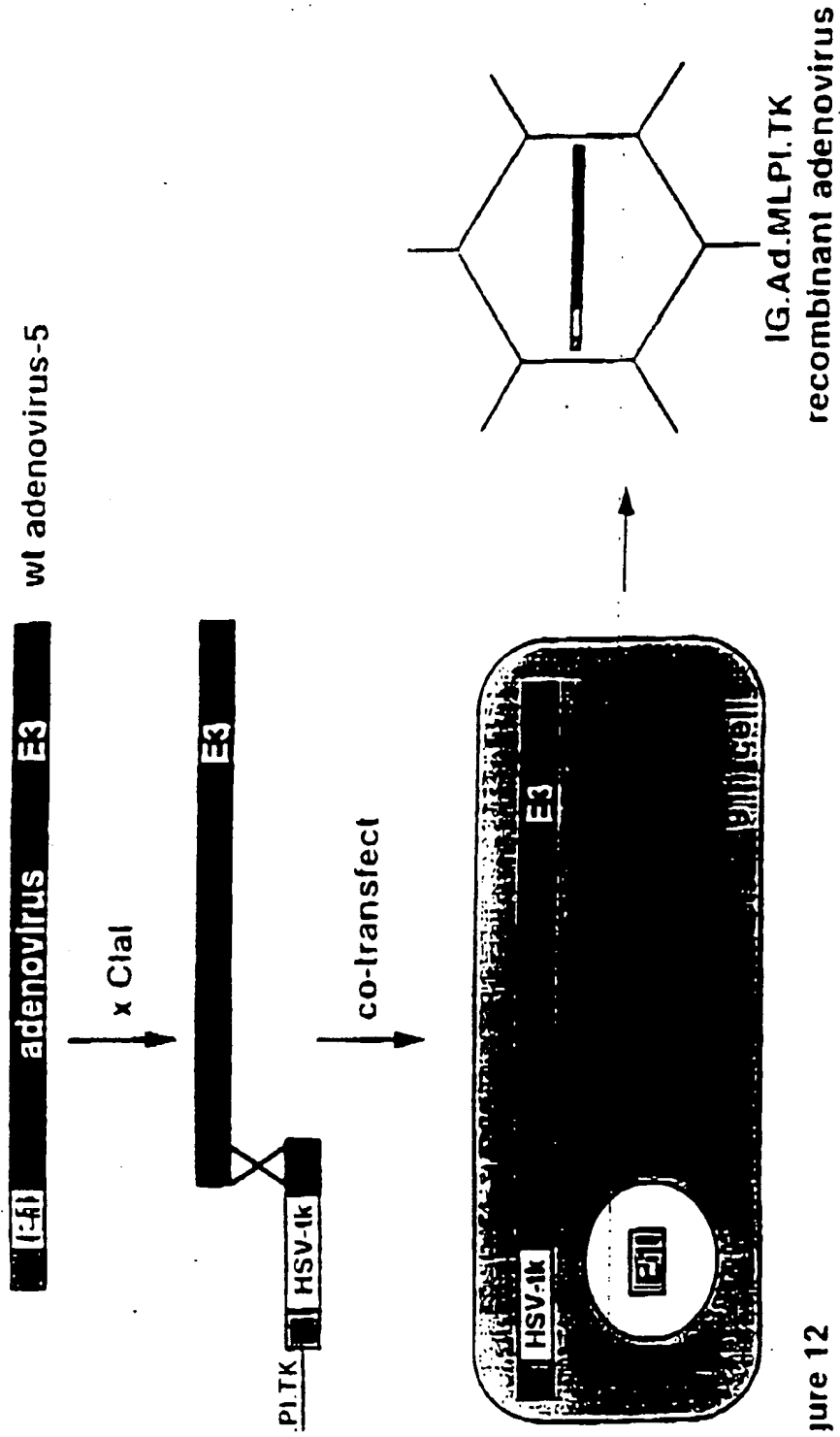
Figure 11a
Packaging system based on primary cells

New recombinant adenoviruses and packaging constructs without sequence overlap



Figure 11.b
Packaging system based on established cell lines: transfection with E1a and selection with G418

Generation of recombinant adenovirus



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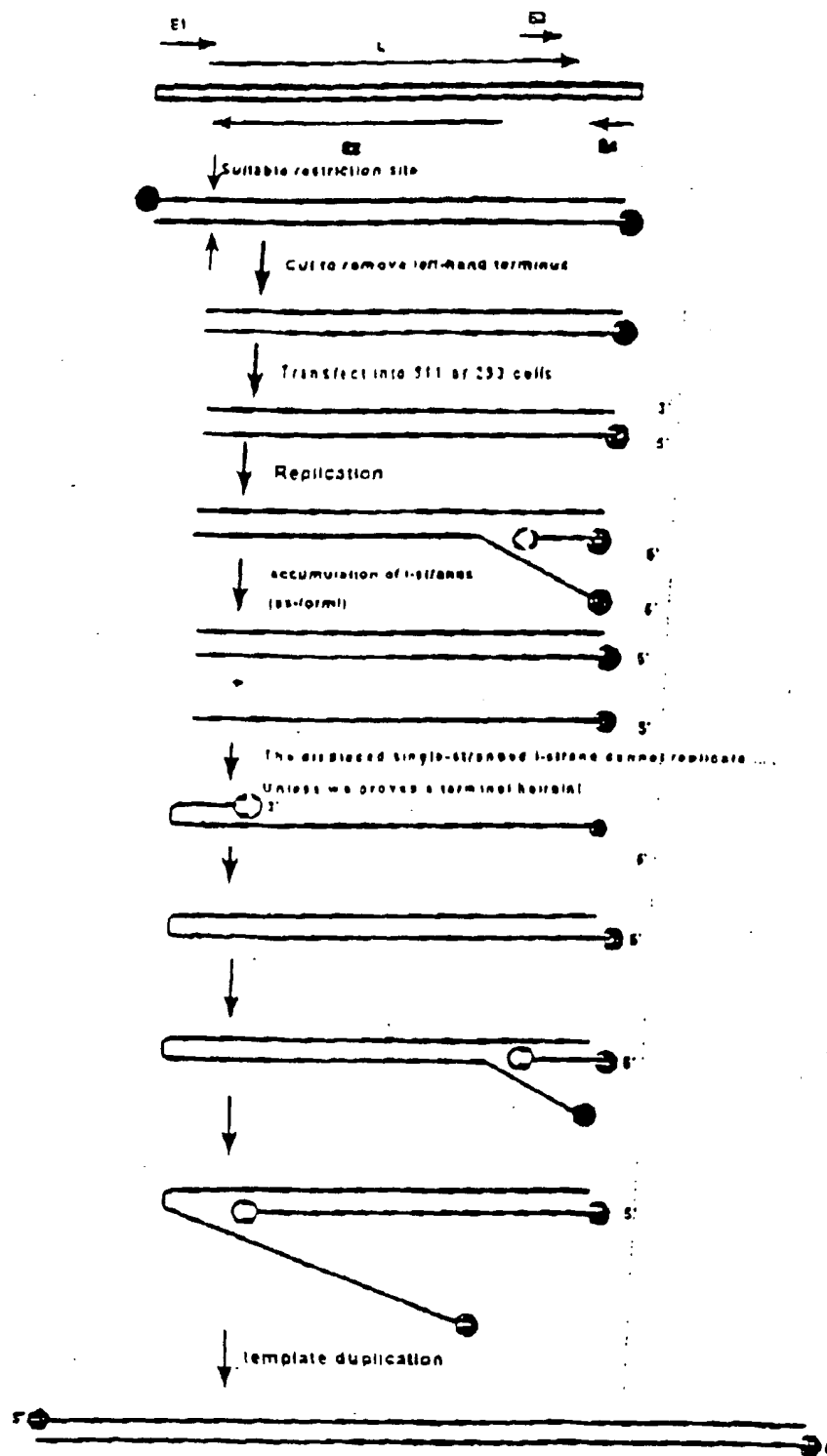


Figure 13

Replication of Adenovirus

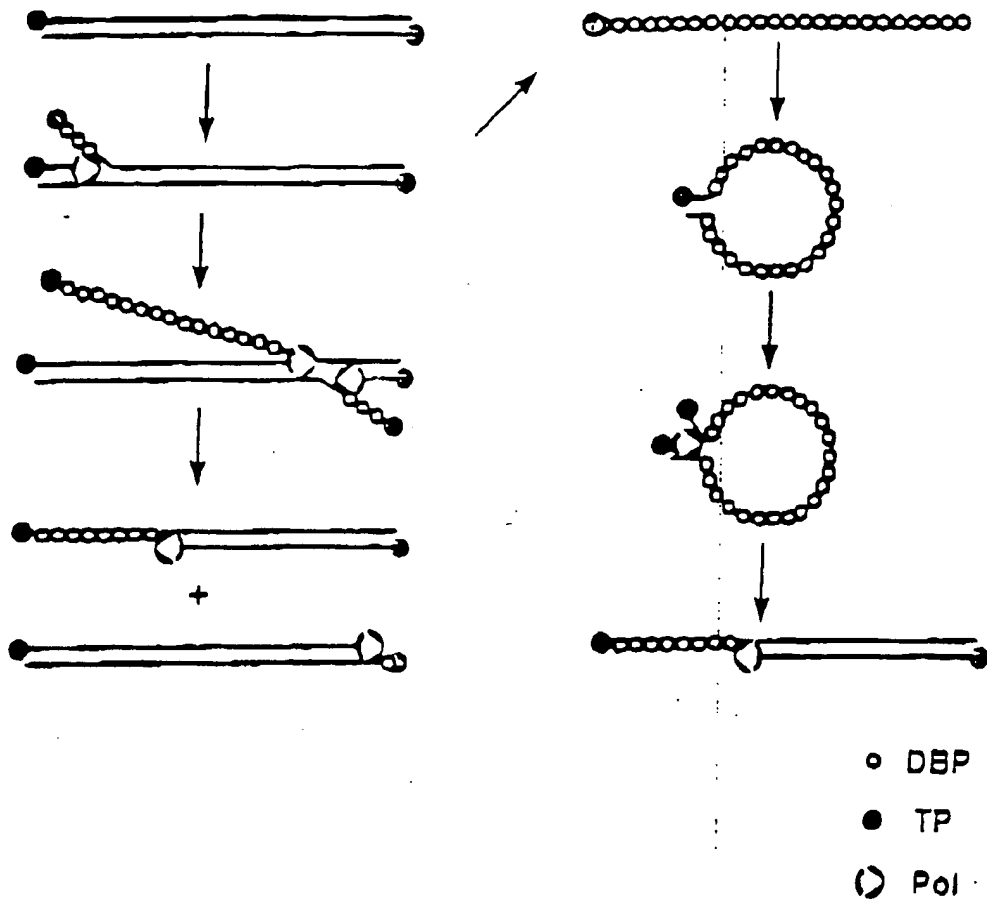


Figure 14

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5'-GTACACTGACCTAGTGCCGCGCGCGGCA
      ||||| A
3'-GATCAGCGCGCGCGCGGCA

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Fig 15. The potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequences used in these studies. Restriction with the restriction endonuclease *Asp*718I of plasmid pICLha₂, containing the annealed oligonucleotide pair HP/asp1 en HP/asp2 will yield a linear double-stranded DNA fragment. In cells in which the required adenovirus genes are present, replication can initiate at the terminus that contains the ITR sequence. During the chain elongation, the one of the strands will be displaced. The terminus of the single-stranded displaced- strand molecule can adopt the conformation depicted above. In this conformation the free 3'-terminus can serve as a primer for the cellular and/or adenovirus DNA polymerase, resulting in conversion of the displaced strand in a double-stranded form.

Figure 15

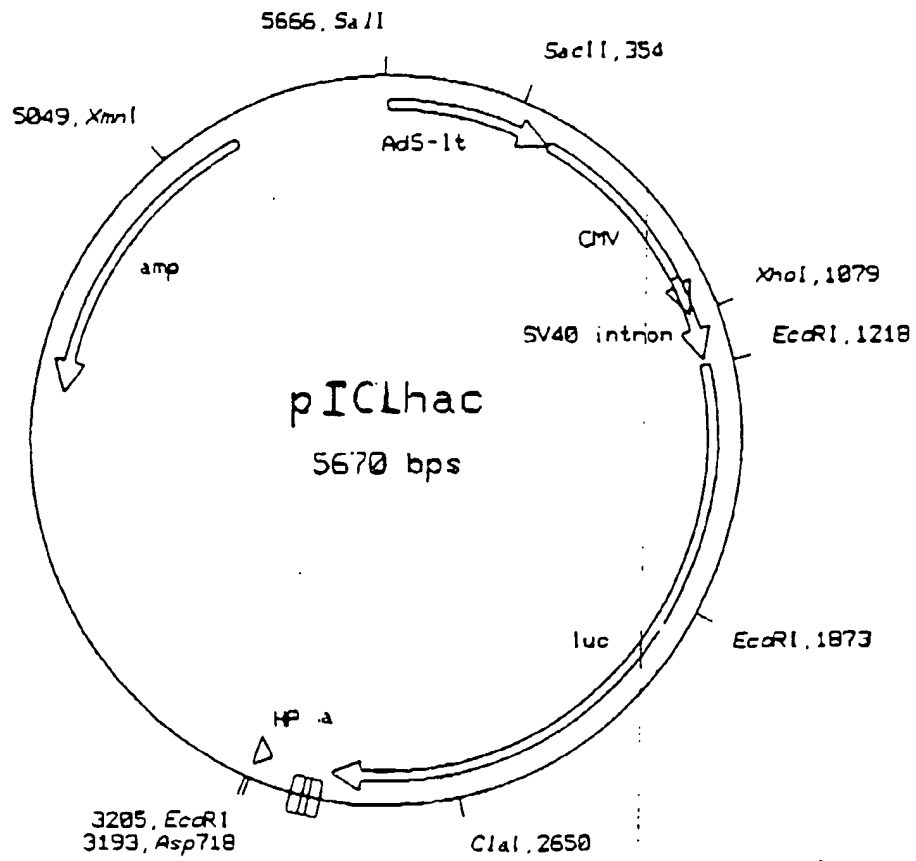


Fig. 16

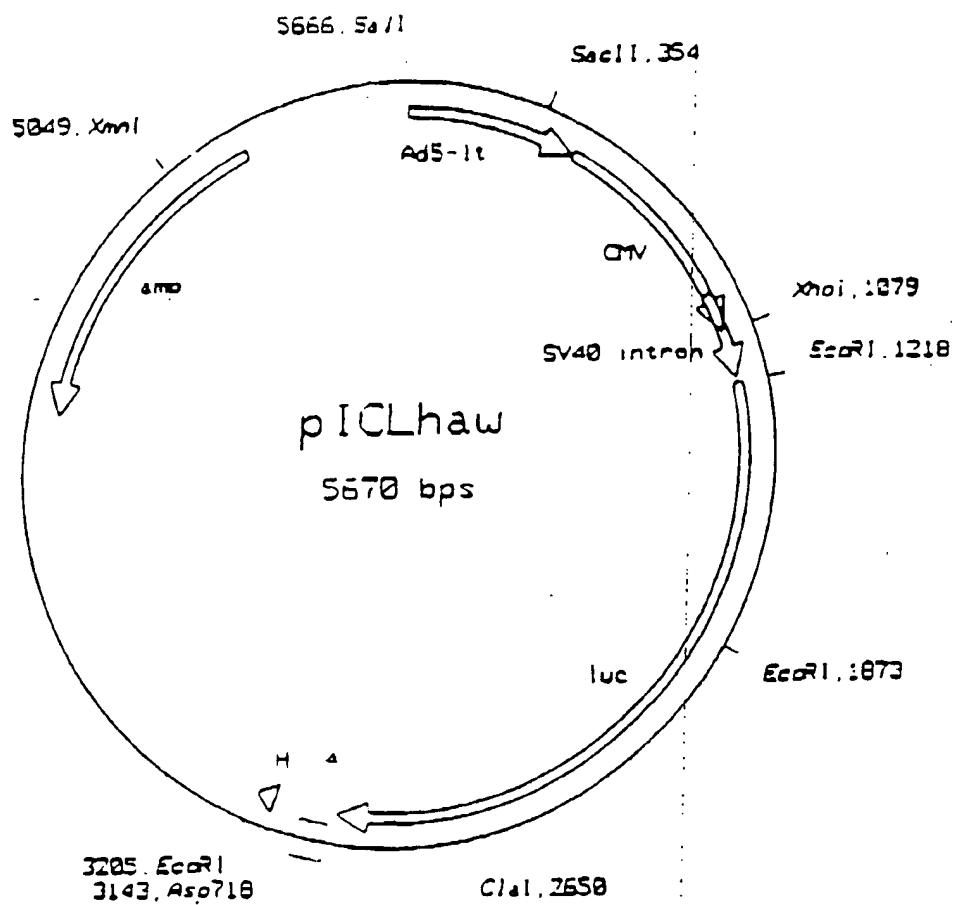


Figure 17

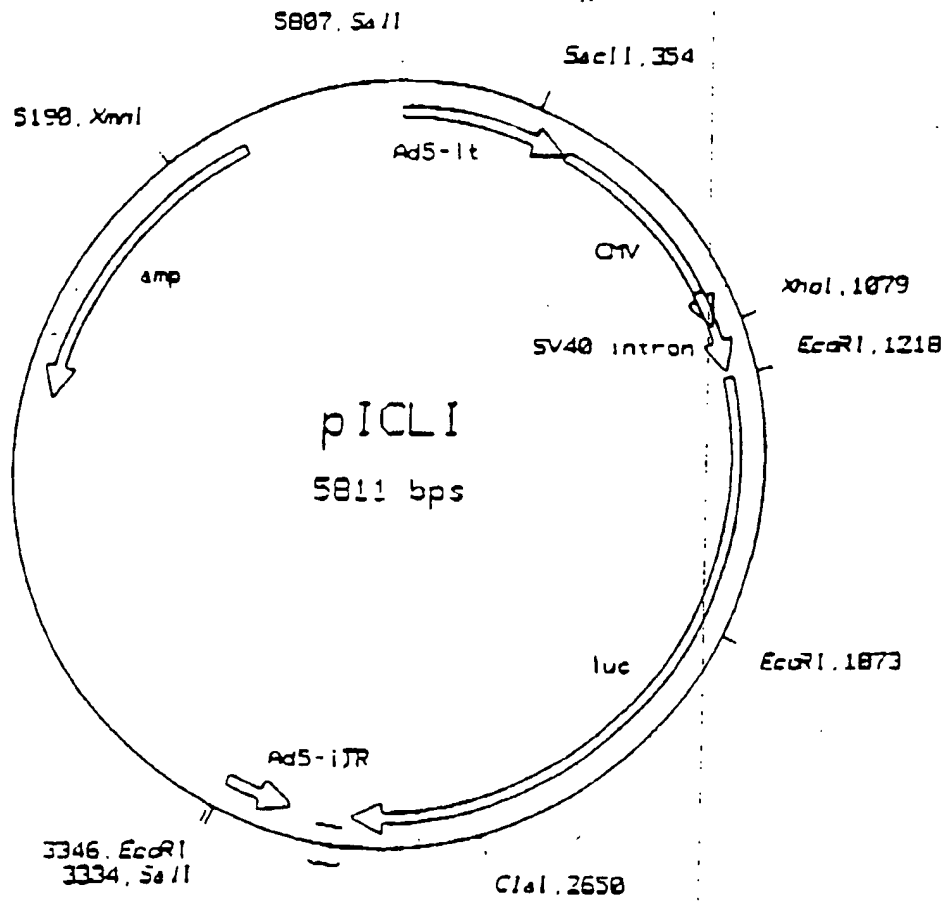


Figure 18

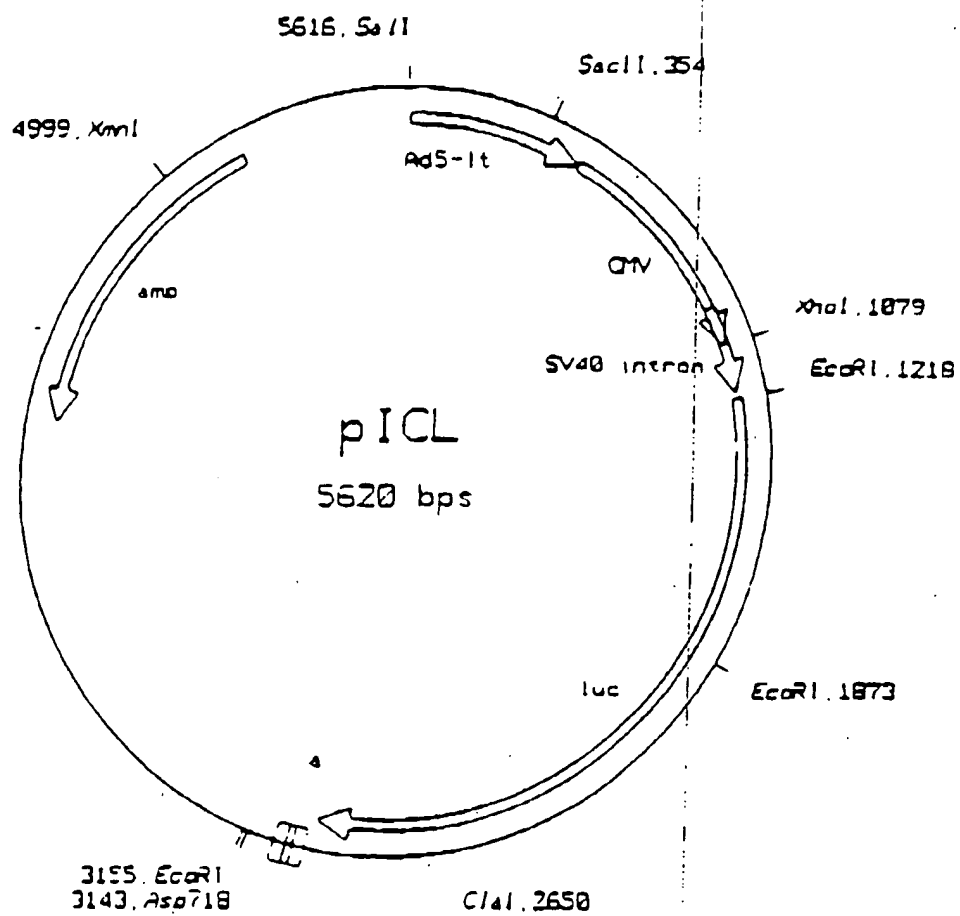


Figure 19

Figure 20: Cloned adenovirus fragments

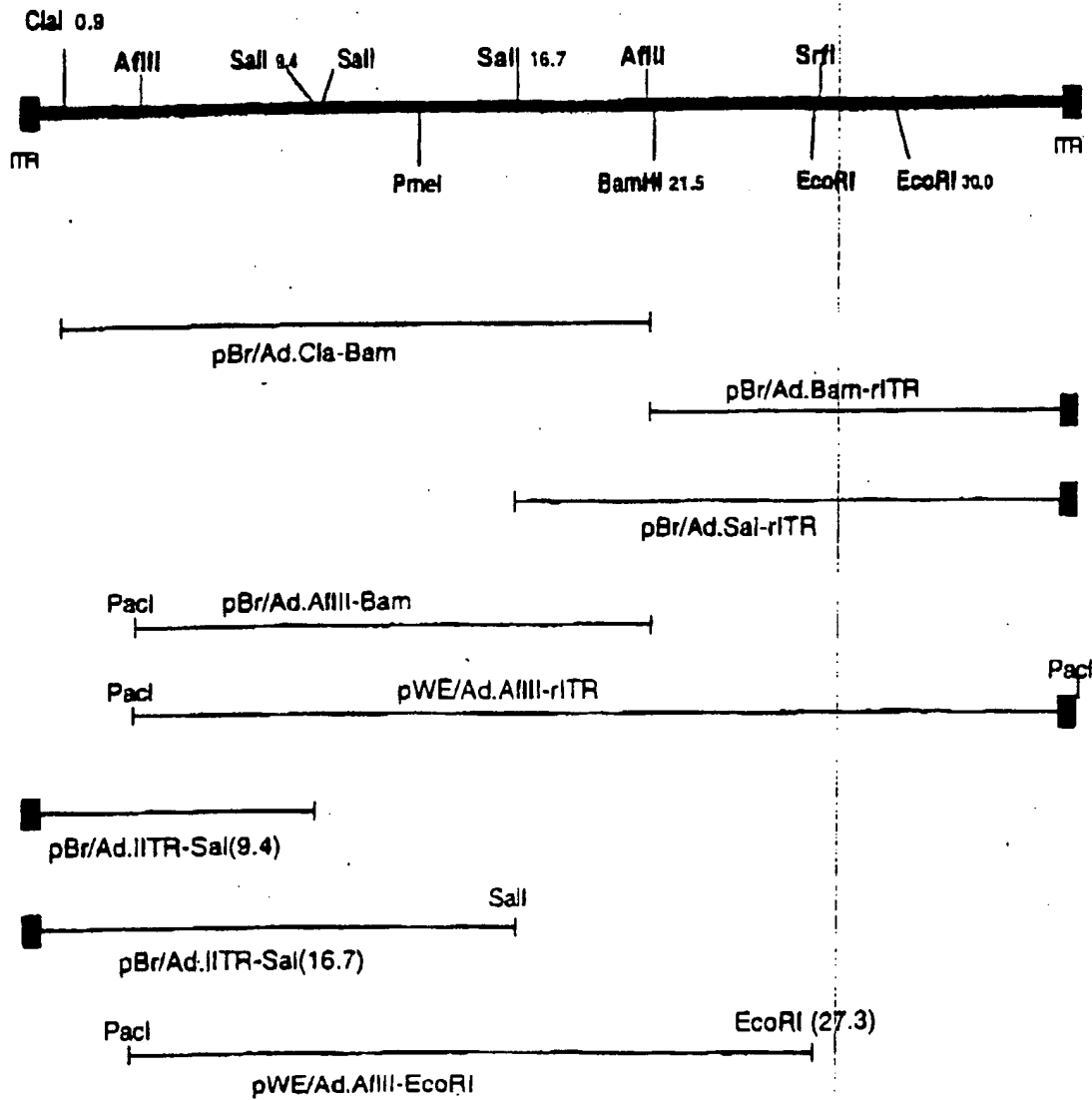
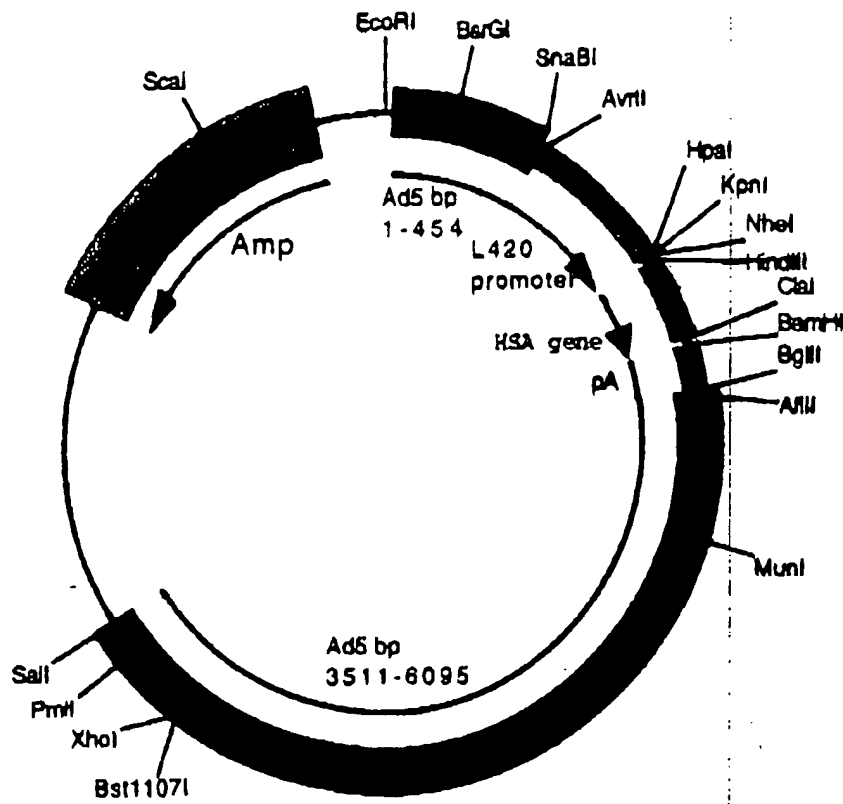


Figure 21: Adapter plasmid pAd5/L420-HSA



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Figure 22: Adapter plasmid pAd5/CLIP

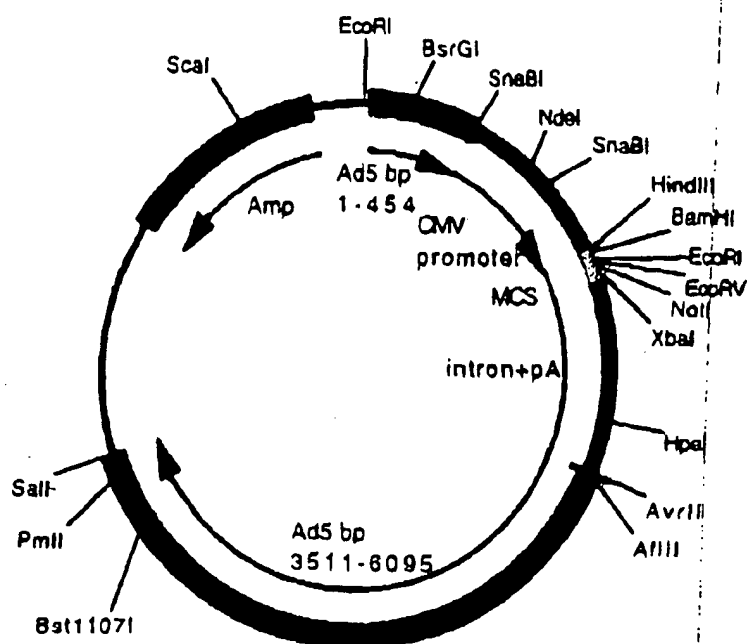


Figure 23: Generation of recombinant adenoviruses

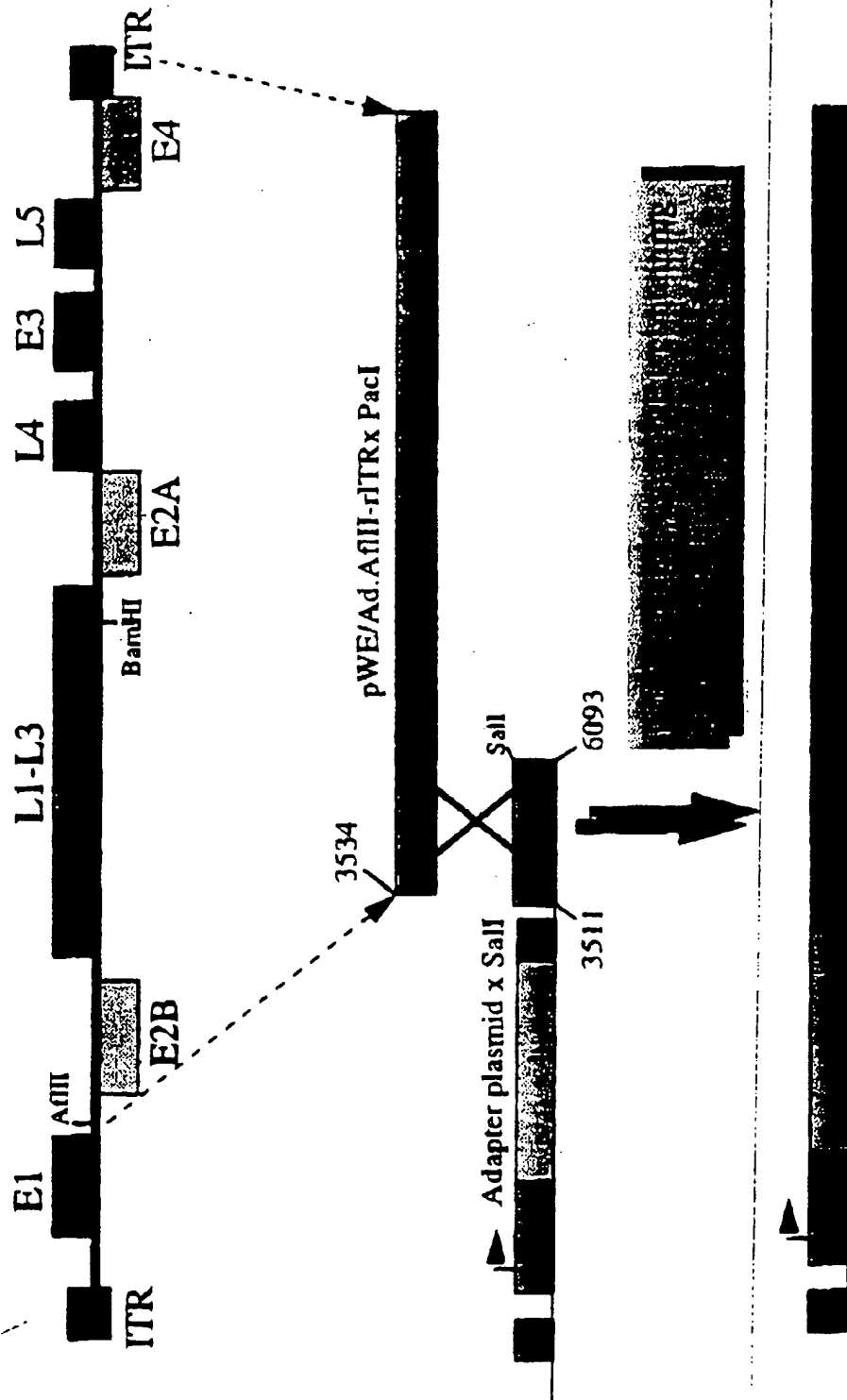


Figure 24: Minimal adenovirus vector pMV/L420H

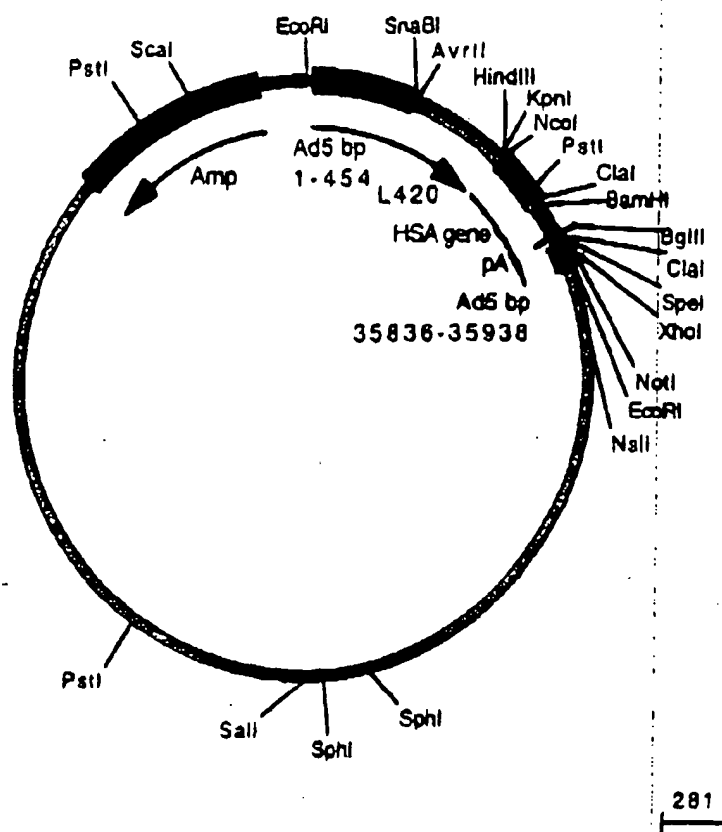


Figure 25: Construction of pWE/AdΔ5'

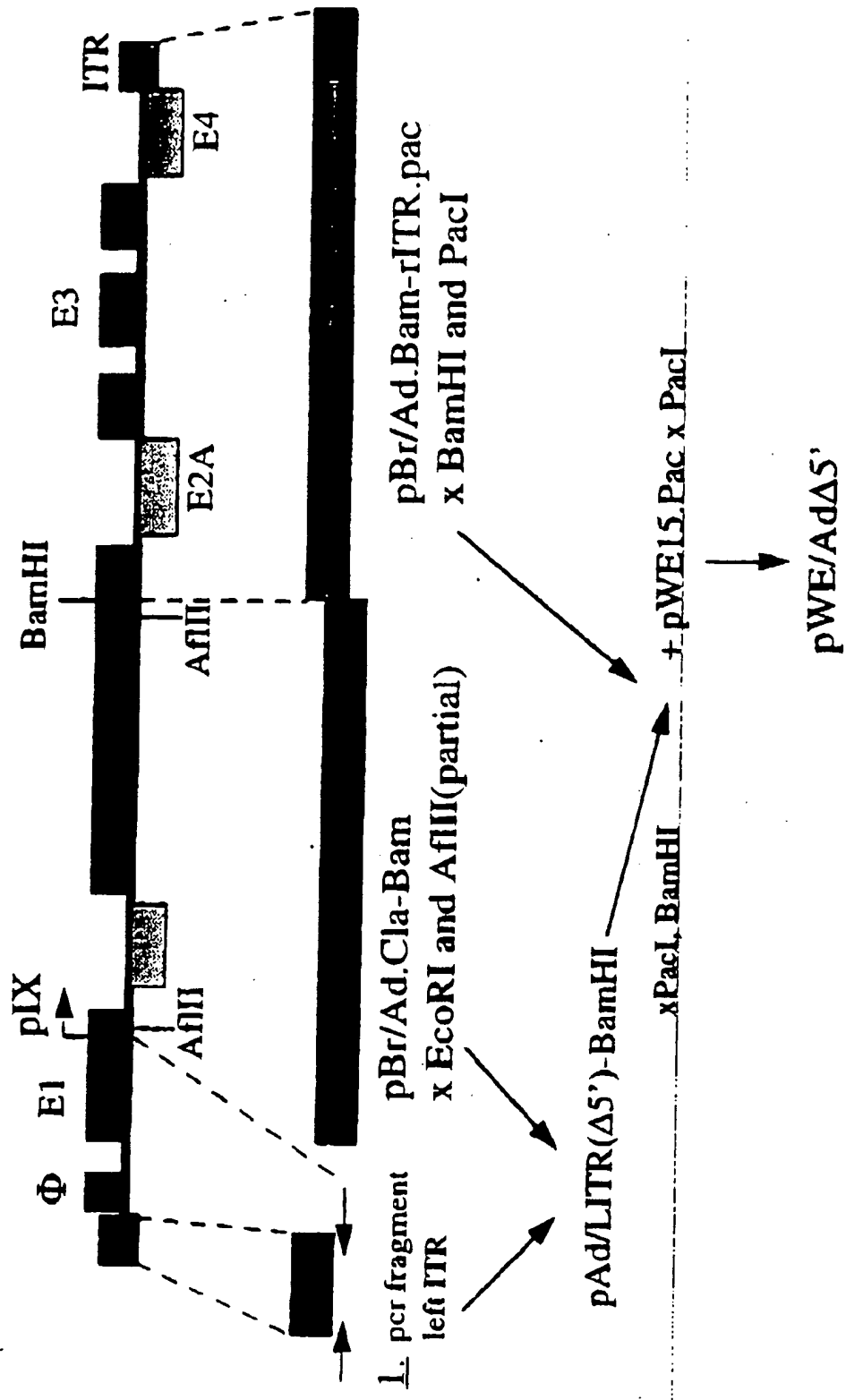
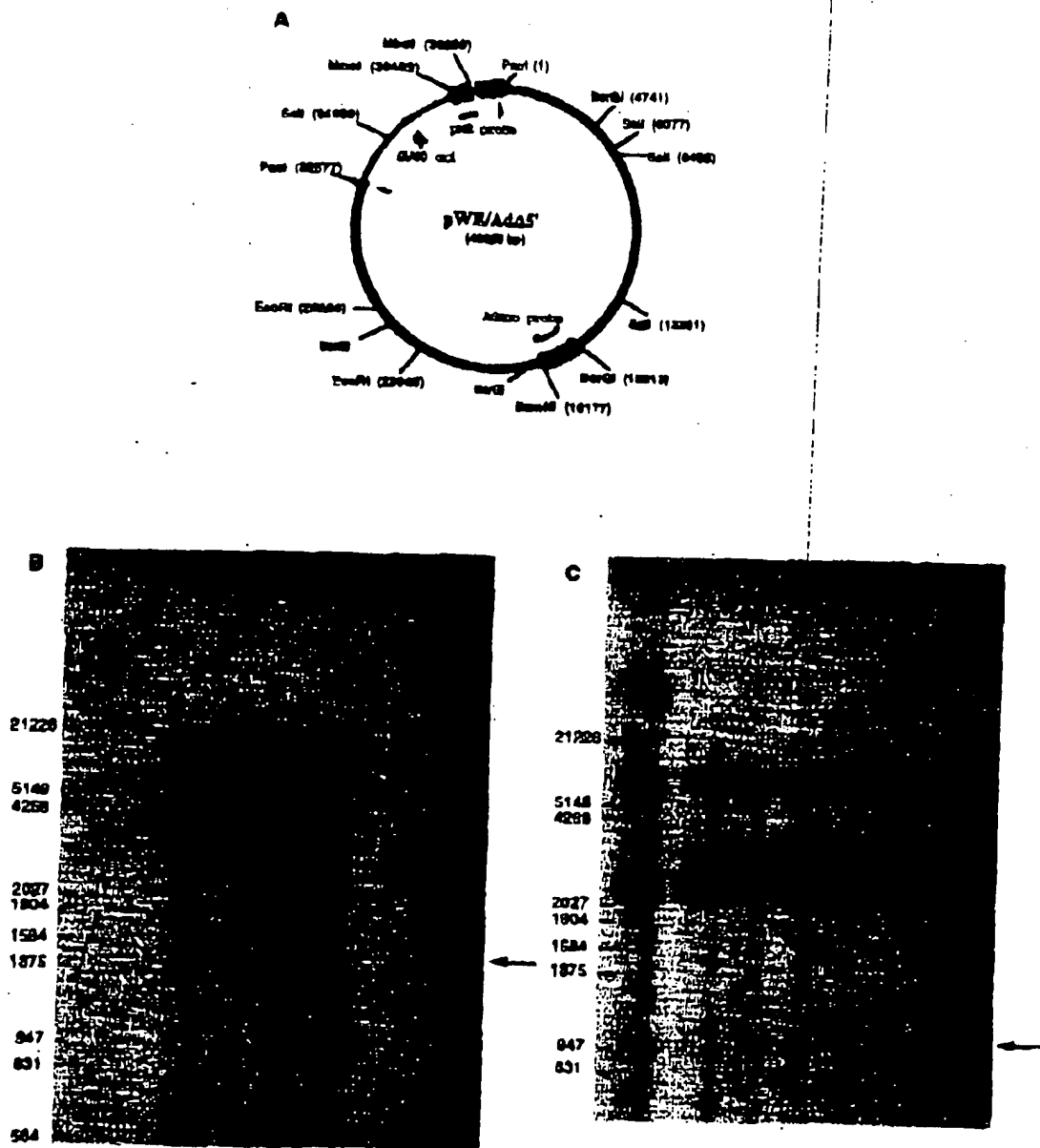


Figure 26



(19)



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(11)

EP 0 955 373 A3

(12)

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(54) **Packaging systems for human recombinant adenovirus to be used in gene therapy**

(57) The invention discloses novel means and methods for the generation of adenovirus vector. One method of the invention entails a method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of com-

bining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof. The invention further discloses nucleic acid molecules for generation adenovirus vectors.

EP 0 955 373 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 20 1278

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 96 17070 A (TRANSGENE SA ;CHARTIER CECILE (FR); DEGRYSE ERIC (FR)) 6 June 1996 (1996-06-06) * page 9, line 6 - page 12, line 26; claims 1-3,15; figure 1 *	1,3,4,7, 15,16	C12N15/86 C07K14/075 C12N5/10 C12R1/01 C12N15/00 C12N7/00 A61K48/00
X	OKADA, T. ET AL.: "Efficient directional cloning of recombinant adenovirus vectors using DNA-protein complex" NUCLEIC ACIDS RESEARCH, vol. 26, no. 8, 15 April 1998 (1998-04-15), pages 1947-1950, XP002121030 OXFORD GB * the whole document *	1-3	
X,D	CHARTIER, C. ET AL.: "Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli." JOURNAL OF VIROLOGY., vol. 70, no. 7, July 1996 (1996-07), pages 4805-4810, XP002059961 ICAN SOCIETY FOR MICROBIOLOGY US * the whole document *	1,3,4,7	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N C07K
X,D	CROUZET, J. ET AL.: "Recombinational construction in Escherichia coli of infectious adenoviral genomes" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 94, no. 4, February 1997 (1997-02), pages 1414-1419, XP002109110 NATIONAL ACADEMY OF SCIENCE. WASHINGTON., US ISSN: 0027-8424 * figure 1 *	1,3,4	
-/--			
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 1 November 1999	Examiner CHAMBONNET, F
CATEGORY OF CITED DOCUMENTS X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background O: non-written disclosure P: intermediate document		T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &: member of the same patent family, corresponding document	

EPC FORM 1503 (03.02) (P24C01)



European Patent
Office

Application Number

EP 99 20 1278

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely

see sheet B

- ☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-17



European Patent
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EUROPEAN SEARCH REPORT

Application Number
EP 99 20 1278

DOCUMENTS CONSIDERED TO BE RELEVANT				
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)	
X	HE, T.C. ET AL.: "A simplified system for generating recombinant adenoviruses" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 95, no. 5, 3 March 1998 (1998-03-03), pages 2509-2514, XP002109113 WASHINGTON US * the whole document *	1,3,4,7, 15		
A	WO 97 00326 A (FALLAUX FRITS JACOBUS ;UNIV LEIDEN (NL); BOUT ABRAHAM (NL); EB ALE) 3 January 1997 (1997-01-03) * page 29, line 31 - page 30, line 4 * * page 37, line 10 - line 20; claims *	11-14		
A	EP 0 707 071 A (INTROGENE BV) 17 April 1996 (1996-04-17) * the whole document *	1		
A	VOLKERT, F.C. & YOUNG, C.S.H.: "The genetic analysis of recombination using adenovirus overlapping terminal DNA fragments" VIROLOGY, vol. 125, no. 1, February 1983 (1983-02), pages 175-193, XP002121031 ORLANDO US * page 175, column 2, paragraph 3 - page 176, column 2, paragraph 2 *	1,3		TECHNICAL FIELDS SEARCHED (Int.Cl.6)
P,X	WO 98 32860 A (BAXTER INT) 30 July 1998 (1998-07-30) * figures 4-6 *	1,2,4,5, 7,12		
----- The present search report has been drawn up for all claims				
Place of search THE HAGUE		Date of completion of the search 1 November 1999	Examiner CHAMBONNET, F	
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document		

EPO FORM 1503 (31.03.92 (patent))



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LACK OF UNITY OF INVENTION
SHEET B

Application Number

EP 99 20 1278

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-17

a method for generating an adenovirus vector comprising welding together two nucleic acid molecules whereby said molecules comprise partially overlapping sequences capable of combining with each other allowing the generation of a physically linked nucleic acid comprising at least two functional adenovirus inverted terminal repeats, a functional encapsulation signal and a nucleic acid of interest or functional parts, derivatives and/or analogues thereof;

2. Claims: partially 18 21 22 28 33-35 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No.P97082122 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

3. Claims: partially 18 21 22 28 33-35 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No.P97082119 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

4. Claim : partially 18 28 33 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No.P97082117 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

5. Claim : partially 18 28 33 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No.P97082114 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

6. Claims: partially 18 21 22 28 33-35 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No.P97082120 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

7. Claim : partially 18 28 33 47 53 57 58



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

A recombinant nucleic acid deposit at the ECACC under No. P97082121 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

8. Claims: partially 1, 21 22 28 33-35 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No. P97082116 or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

9. Claim : partially 18 28 33 47 53 57 58

A recombinant nucleic acid deposit at the ECACC under No. P97082115 and P97082118, or a functional part, derivative and/or analogue thereof; cells comprising said nucleic acid;

10. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pWE/Ad.AflIII-EcoRI; cells comprising said nucleic acid;

11. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pAd5/CLIP; cells comprising said nucleic acid;

12. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pAd5/L420-HAS; cells comprising said nucleic acid;

13. Claim : partially 19 33 28 47 53 57 58

A recombinant nucleic acid pBs.Eco-Eco/ad5deltaHIII deltagp19KdeltaXbaI; cells comprising said nucleic acid;

14. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pMV/L420-H; cells comprising said nucleic acid;



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

15. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pMV/CMV-LacZ; cells comprising said nucleic acid;

16. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pWE/Ad.delta5'; cells comprising said nucleic acid;

17. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pWE/AAV.delta5'; cells comprising said nucleic acid;

18. Claim : partially 19 28 33 47 53 57 58

A recombinant nucleic acid pWE/Ad-H; cells comprising said nucleic acid;

19. Claim : 20 and partially 28 33 47 53 57 58

A recombinant nucleic acid comprising adenovirus derived nucleotides 1-454 and adenovirus nucleotides 3511-6095; cells comprising said nucleic acid;

20. Claims: 23-24 and partially 28 33 47 53 57 58

A recombinant nucleic acid comprising a nucleotide sequence based on or derived from an adenovirus wherein said nucleotide comprises sufficient adenovirus sequences necessary for replication and capsid expression, wherein said nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus; cells comprising said nucleic acid;

21. Claims: 25-27 and partially 28 33 47 53 57 58

A recombinant nucleic acid comprising a nucleotide sequence based on or derived from an adenovirus wherein said nucleotide comprises a sequence for adenovirus-independent replication and sufficient adenovirus sequences necessary for replication, wherein said nucleotide sequence comprises at least a deletion of the E1 region and encapsulating signal of said adenovirus; cells comprising said nucleic acid;



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely.

22. Claims: 29-32 and partially 19 33-35 47 54 57 58

An adapter plasmid comprising a nucleotide sequence based on or derived from an adenovirus wherein said nucleotide sequence comprises in operable configuration at least one Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination and the generation of a replication-defective, recombinant adenovirus genome, in so far as it is not used in method of the first invention; cells comprising said adapter plasmid; uses thereof.

23. Claims: partially 34 35 54-56

A method for the generation of recombinant adenovirus, having an E1 deletion and a gp19k deletion, comprising the steps of:
growing a cell comprising adenovirus complementing sequences transfected with
i) an adapter plasmid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises in operable configuration one functional Inverted Terminal Repeat, one functional encapsulating signal and adenoviral sequences which allow for homologous recombination leading to the generation of a replication-defective, recombinant adenovirus genome, in a cell into which said adapter plasmid is transferred and having no E1 region sequences, and
ii) a recombinant nucleic acid comprising at least one second nucleotide sequence based on or derived from an adenovirus, wherein said at least one second nucleotide sequence comprises one Inverted Terminal Repeat, and sufficient adenoviral sequences for replication and a partial overlap with said plasmid adapter, wherein said at least one second nucleotide sequence comprises a deletion of at least the E1 region, encapsulating signal and gp19k sequences, wherein said complementing sequences, said first nucleotide sequence and at least one second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus having an E1 and a gp19k deletion is generated.

24. Claims: 36 and partially 37-39

A method for the generation of recombinant adenovirus, comprising the steps of:
growing a cell comprising adenovirus complementing sequences



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

transfected with

- i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and
- ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises a sequence for viral replication, and sufficient adenovirus sequences necessary for replication, wherein said second nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus; wherein said complementing sequences, said first recombinant nucleic acid and said second recombinant nucleic acid has no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated.

25. Claims: partially 37-39

A method for the generation of recombinant adenovirus, comprising the steps of:
growing a cell comprising adenovirus complementing sequences transfected with

- i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and
- ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises a sequence for adenovirus-independent replication, and sufficient adenovirus sequences necessary for replication, wherein said second nucleotide sequence comprises a deletion of at least the E1 region and encapsulating signal of said adenovirus; wherein said complementing sequences, said first recombinant nucleic acid and said second recombinant nucleic acid has no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated; as far as not covered by the method of subject 24.

26. Claims: 40-45 49-52



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

A recombinant defective adenovirus comprising a genome based on or derived from an adenovirus, wherein said genome comprises at least a functional encapsulating signal and two functional Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said genome comprises no functional adenovirus genes and has no overlapping sequences which allow for homologous recombination leading to replication competent virus, in a cell into which said replication defective adenovirus is transferred; non human cells comprising such a genome.

27. Claim : 46

A method for the generation of recombinant adenovirus, comprising the steps of:
growing a cell comprising adenovirus complementing sequences and

i) a first recombinant nucleic acid comprising a first nucleotide sequence based on or derived from an adenovirus, wherein said first nucleotide sequence comprises a functional encapsulating signal and two Inverted Terminal Repeats or functional fragments or derivatives thereof, and wherein said first recombinant nucleic acid has no functional adenovirus genes and
ii) a second recombinant nucleic acid comprising a second nucleotide sequence based on or derived from an adenovirus, wherein said second nucleotide sequence comprises at least all adenovirus sequences, or functional fragments or derivatives thereof, necessary for replication and capsid gene expression, and a complementary sequence to an upstream part of the same strand of said nucleic acid, wherein said complementary sequence can base-pair with said upstream part so that it functions as a start-site for a nucleic acid polymerase, wherein said second nucleotide sequence comprises a deletion of one Inverted Terminal Repeat, the E1 region and the encapsulating signal of said adenovirus; wherein said complementing sequences, said first nucleotide sequence and said second nucleotide sequence have no overlapping sequences which allow for homologous recombination leading to replication competent virus, under conditions whereby recombinant adenovirus is generated;

28. Claim : 48

A method for the replacement of a defective gene in a host cell genome comprising the step of:
growing said host cell with a recombinant nucleic acid derived from a replication defective adenovirus, comprising a functional version or part thereof of said defective gene in said host cell genome is replaced.



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The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

29. Claim : partially 55 and 56

A recombinant defective adenovirus comprising a genome based on or derived from an adenovirus, wherein said genome comprises a first deletion in an E1 region and a second deletion in a gp19k region; as far as not covered by the subject number 23.

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 99 20 1278

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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01-11-1999

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